TUMOURIGENESIS

AND THE

PHOSPHOINOSITIDE PATHWAY

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A thesis submitted for the degree
of.philosophy of the Australian National
University.

May 1988

STATEMENT

THE STUDY DESCRIBED IN THIS THESIS WAS CARRIED OUT ENTIRELY BY THE CANDIDATE

Amanda Crozzi

TO MY PARENTS
WITH LOVE

ACKNOWLEDGEMENTS:

THANK-YOU to my three supervisors, Dr. Michael Banyard, Dr. Nick Hunt and Dr. Terry Walsh. To Mike for his guidance and enthusiasm, to Nick for his constant support and critical appraisal and to Terry for his practical and invaluable advice. All three gave so willingly of their time and experience.

To Prof. Peter Doherty as Head of the Department of Experimental Pathology, the John Curtin School of Medical Research, and as the tenured member of my supervisory committee, for his continued interest.

To Dr. Ian Buckley for his professional and moral support.

To Dr. Bruce Kemp for his gift of the Protein kinase C substrate GS(1-12) and for his willingness to demonstrate experimental techniques. Thank-you, also in this respect to Colin House, Dr. Marjorie Dunlop and Damian Myers.

To Dr. Trevor Biden and Dr. Ross Tellam in their advisory capacity both official and unofficial.

To Mr. Ross Cunningham for his statistical advice and Mr. Charles Claudianos in his operation of the HPLC facility.

To Dr Geeta Chaudri for her mastery of the MacIntosh in the production of figures and diagrams.

To everyone in the Department of Experimental Pathology and throughout the School for making this three years a "time to remember".

To the Commonwealth Government of Australia and the Australian National University for providing financial support.

And last but certainly not least, to my wonderful typist Mrs Judy Bradley of CANWORDS for her infinite patience.

PUBLICATIONS

Carozzi, A.J., Banyard, M.R.C., Hunt, N.H. and Tellam, R. (1987) "The Role of the Phosphoinositide Pathway in the Progression from the Transformed to the Tumourigenic Phenotype in the HeLa/Fibroblast Somatic Cell Hybrid System" Proceedings of the Australian Biochemical Society, 19: 62.

ABSTRACT

Several aspects of the phosphoinositide signal transduction system were examined in transformed and tumourigenic HeLa x fibroblast human somatic cell hybrids. The advantage of this cell system is that both transformed and tumourigenic cell lines can be derived from the one heterokaryon and, therefore, any noted differences between the pairs of cells is highly likely to be associated with the expression of the tumourigenic phenotype in vivo.

The incorporation of inositol into the total phosphoinositide pools in serum-stimulated hybrids was examined and was found to be equivalent in transformed and tumourigenic cells. Therefore, it was inferred that the rate of breakdown of the phosphoinositides did not differ between the transformed and tumourigenic hybrids. A comparison of the normal and tumourigenic cell lines representing the hybrid parents, also indicated no difference in the turnover rate of the phosphoinositide pools. However, the absolute size of the steady state pools differed markedly between the parental lines.

Thorough examination of the steady state levels of the phosphoinositides in the transformed and tumourigenic hybrids indicated that there was no difference in the pool sizes within two of the hybrid pairs but that one hybrid pair and the parental lines did display a statistically significant difference in the size of their pools, with the levels in the non-tumourigenic cells exceeding those in their tumourigenic counterparts. However, when results were expressed per unit

area of membrane rather than per cell, it was found that in each case the tumourigenic cells had slightly higher steady state levels of phosphoinositides than their non-tumourigenic partners. Nevertheless, the variation was not significant by statistical analysis.

It was found that the ratio of phosphatidylinositol (4,5)bisphosphate to phosphatidylinositol did not differ between the transformed and tumourigenic cell hybrids, supporting the contention that the findings concerning total pool sizes mirror that proportion of the phosphoinositide pool directly relevant to signal transduction. However, more direct evidence, that there was no change in phosphatidylinositol(4,5)-bisphosphate hydrolysis between the cell types, was obtained by monitoring the steady state levels of the inositol phosphates in the hybrid pairs. It was found that there was no consistent difference in the levels of any of the inositol phosphates, with the levels of the proposed second messenger inositol(1,4,5)trisphosphate being similar in the transformed and tumourigenic cells. Therefore, it was concluded that the expression of the tumourigenic phenotype in this cell system is not associated with an alteration in phosphoinositide metabolism under standard tissue culture conditions.

To examine the possibility that variations between the hybrid pairs occur in the phosphoinositide pathway at stages subsequent to lipid hydrolysis, the properties of protein kinase C were investigated in the membrane and cytosolic fractions from the different cell types. It was found that the tumourigenic hybrids and the HeLa parent had

substantially less PKC activity associated with their membranes than did the non-tumourigenic cells. Total cellular activities were also decreased in the tumourigenic lines but not to the same extent as the membrane-associated activities, a finding which may have functional implications since it is the membrane-bound form of the enzyme which is involved in signal transduction.

Moreover, an independent measure of the levels of protein kinase C in the different cell types, determined by examining the levels of phorbol diester binding in intact cells, further indicated that the tumourigenic cell lines had reduced amounts of the enzyme. Therefore, it was concluded that a reduction in the activity of protein kinase C, particularly that bound to the membrane, is associated with the expression of the tumourigenic phenotype.

The implications of these findings were considered in relation to the involvement of a deregulation of signal transduction in the maintenance of the tumourigenic phenotype.

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CHAPTER 1:

INTRODUCTION

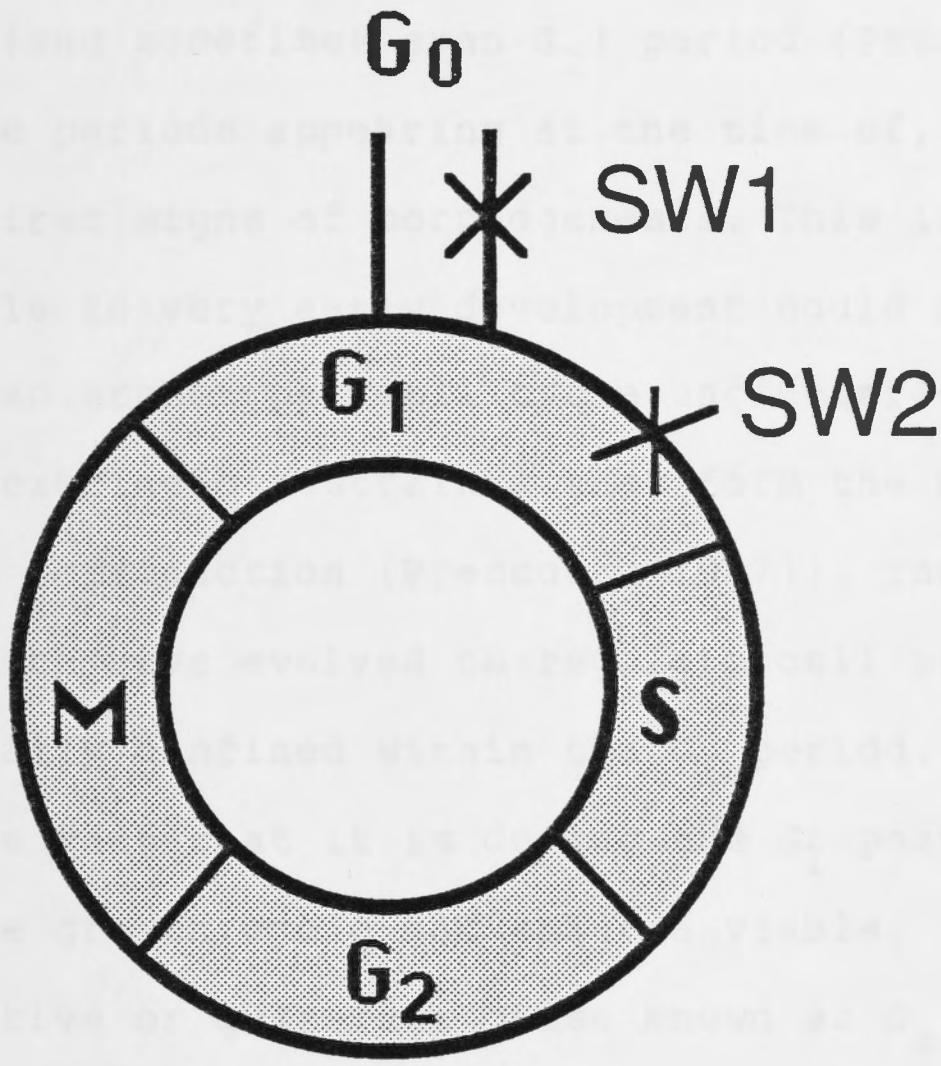
One of the greatest challenges of cell biology involves determining the sequence of events which direct a cell to divide. It is only when this sequence is known that one can fully assess how cellular growth is regulated and therefore gain some clearer insight into how it can be de-regulated when a cell becomes malignant.

1.A CELL CYCLE

The majority of actively dividing higher animal and plant cells have four stages in their growth cycle (see diagram 1.1). The S phase designates the period occupied by DNA replication. This is separated from the visible start of nuclear division (M phase) by a period during which most cell growth occurs, known as the G_2 phase. Cell division is then separated from the next round of DNA replication in most cells by another temporal gap known as the G_1 period (for reviews see Prescott (1987); Pardee, (1978)).

Not all cells, however, have a detectable G_1 period. For example, it is missing from at least three mammalian tissue culture lines [Chinese hamster line DON, a mouse teratocarcinoma (cited in Prescott (1982)) and the hamster line V79-8 (Liskay and Prescott (1978)] and rapidly dividing cells in the erythropoietic series do, apparently, at some stage of differentiation, lack a G_1 phase (Alpen and

Figure 1.1 The Cell Cycle



Stages of the Growth cycle

- S = DNA replication.
- G_2 = Gap 2 during which most cell growth occurs.
- M = Cell division.
- G_1 = Gap 1 during which regulation of the cell cycle occurs. This is the stage where the decision to remain within the cell cycle is made.
- G_0 = Quiescent state when the cells have left the growth cycle.

The length of each stage in the cell cycle is variable according to cell type.

For SW1 and SW2 see section 1.D.1.

Johnston (1967)). In addition, the embryonic development of animals in general, begins with a series of cell divisions lacking a G_1 (and sometimes even G_2) period (Prescott (1987)), these periods appearing at the time of, or just before, the first signs of morphogenesis. This is important since the cycle in very early development could be considered to represent an archetypal cell cycle uncluttered by the intrinsic or extrinsic restraints that form the mechanisms that regulate cell reproduction (Prescott (1987)). Therefore, the key events which have evolved to regulate cell proliferation may be temporally confined within the G_1 period. In support of this is the fact that it is during the G_1 phase that cells can escape the growth cycle and enter a viable, non-proliferative or quiescent phase known as G_o (Pardee 1978). Arrest in the G_1 phase and subsequent entry into G_o is generated in cultured normal cells by such factors as serum deprivation (Holley (1975)), removal of essential nutrients (Ley and Tobey 1970)) or spatial constraints leading to contact inhibition (Lieberman and Glaser (1981)).

The induction of quiescence by deprivation of growth factors is considered to be an important element in the regulation of cell proliferation in vivo (Holley (1975)). Much information has been collected over the last 20 years concerning the way in which a variety of polypeptide growth factors, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and insulin, acting outside the cell, transmit their growth messages to the cell interior. This occurs via interaction of factors with specific

receptor proteins on the cell surface (reviewed in Rozengurt and Collins (1983)). It seems reasonable, therefore, to suppose that growth factors and their receptors, represent externalization of part of the circuitry that operates to regulate G_1 arrest and release, allowing specific regulation of cell proliferation from outside the cell.

The molecular means by which the binding of growth factors to their receptors leads to the transition from the G_o to the G_1 phase, the activation of the G_1 S switch and ultimately, some hours later, to mitosis, remains largely unknown (Pardee (1987)). Some of the early events, occurring within seconds to minutes after growth factor binding, are well documented (Rozengurt (1986); Metcalf et al. (1985)) as are the mechanisms of DNA replication and cell division (Gefter (1975)). However, the means by which receptor occupancy leads to the generation of the early events has only recently been uncovered and the events linking these early changes to DNA replication remains obscure (Pardee (1987)).

1.B EARLY EVENTS ASSOCIATED WITH GROWTH FACTOR BINDING

Several biochemical changes which have been reported to occur within 5 minutes of growth factor binding, are common to a wide range of cells (for reviews see Rosengurt, (1986); Metcalfe et al. (1985); Pouysségur et al. (1982a)). These include:-

1. increased ion fluxes across the plasma membrane
2. elevated intracellular pH
3. altered phosphorylation of a number of cellular proteins

1.B.1 ION FLUXES:

One of the earliest responses elicited by addition of growth factors to quiescent cells, is an increase in the fluxes of Na^+, K^+ and H^+ ions across the plasma membrane. (Rozengurt (1982); Leffert (1982)) It now appears clear that many of these initial ionic events are generated by activation of the Na^+/H^+ antiport (Moolenaar et al. (1982), (1983)) which results in an influx of Na^+ ions into the cell, accompanied by an efflux of H^+ ions. This results in a secondary stimulation of Na^+/K^+ ATPase pump activity which both increases intracellular K^+ and restores the electro-chemical gradient for Na^+ (Smith and Rozengurt (1978)).

In addition to these monovalent fluxes, perhaps the earliest ionic event occurring after mitogenic stimulation (15 sec in fibroblast) is a transient increase in intracellular free Ca^{2+}, accompanied by an efflux of Ca^{2+} ions from the cell (Hesketh et al. (1985); Berridge et al. (1984); Owen and Villereal (1983)). The initial source of these Ca^{2+} ions appears to be from internal stores as the efflux, thought to be mediated through activation of a Ca^{2+}ATPase, can occur even in the absence of external Ca^{2+} (Lopez-Rivas and Rozengurt (1983)). In addition, it appears that there is some maintenance of elevated $[Ca^{2+}]_i$ due to a secondary influx from outside the cell (Joseph et al. (1985)).

Because of the ubiquitous nature of these K^+, Na^+, H^+ and Ca^{2+} fluxes in response to stimulation, much work has centred on examining their potential role as causal

factors in initiating the mitogenic response. The importance of Na^+ to many cells is illustrated by the observation that, if extracellular Na^+ concentrations are reduced below a set level ($\cong$ 40mM), mitogens fail to stimulate DNA synthesis. (Leffert and Koch (1982)). However, it has been shown from studies which have artificially raised $[Na^+]_i$ to levels equivalent to those seen after growth factors stimulation, that an increase in the activity of this ion per se is not a trigger for cell proliferation and it has been proposed that the importance of this ion lies with its role as a counter ion mediating H^+ efflux (Pouysségur et al. (1982)). The role of K^+ ions is also debatable. Studies using ion channel blockers in fibroblasts have indicated that it was possible to prevent growth factor stimulated increases in $[K^+]_i$ or even decrease the G_o arrested $[K^+]_i$ levels without affecting the re-initiation of DNA synthesis (Pouysségur et al. (1982)). However, other studies, again using fibroblasts, from a different species, indicated that a permissive $[K^+]_i$ is necessary for cell division to occur (Lopez-Rivas et al. (1982)).

The rise in intracellular free Ca^{2+} which, in the initial phase at least, appears to be generated independently of the monovalent fluxes (Owen and Villereal (1983)), has been established as a mandatory signal for the action of a number of growth factors (see Berridge (1987a)). Extracellular Ca^{2+} is needed specifically for the $G_o \rightarrow G_1$ and $G_1 \rightarrow S$ transit in many normal cells (Kleine et al. (1986) Milner (1972)) and DNA replication is inhibited in lymphocytes by Ca^{2+}-channel

blockers and the intracellular Ca^{2+} antagonist, TMB-8 (Grier and Mastro (1985)). Moreover, agents which raise $[Ca^{2+}]_i$ have been shown to be mitogenic in some cells systems. (Greene et al. (1976)).

The maintenance of a permissive pH_i, as governed by H^+ ion movements also appears necessary for cell division (Chambard and Pouysségur (1986)) as indicated in the next section.

1.B.2 INTRACELLULAR pH (pH_i):

Control of intracellular pH is complex (reviewed in Roos and Boron (1981)). In bicarbonate free medium, the major ion exchanger responsible for such control is the Na^+/H^+ antiport (for characterization of antiport, see Aronson (1985), Jean et al. (1985), Paris and Pouysségur (1983), Moolenaar (1986)). In such medium, treatment of a variety of cells with their mitogens results in an intracellular alkalinization representing a rise in pH_i of approx. 0.1-0.3 pH units. (Moolenar et al. (1983); Paris and Pouysségur (1984); Mills et al. (1985)). However, even though there is a great deal of evidence to indicate that growth factors activate the Na^+/H^+ exchanger, (Vigne et al. (1985); Mills et al. (1985); Paris and Pouysségur (1984); Vara and Rozengurt (1985); Moolenar et al. (1983)) this does not necessarily imply that the subsequent rise in pH_i is essential for the mitogenic response.

Some of the earliest findings which suggested that pH_i may play a vital role in mitogenesis, came from work

using unfertilized sea urchin eggs. It was shown that incubation of the eggs with weak bases, such as ammonia, could not only raise pH_i, but also mimic many fertilization events including increased protein synthesis, DNA synthesis, polyadenylation of m-RNA and increased K^+ conductance (Whitaker et al. (1982); Mazia (1974)). In addition, studies using fibroblasts have indicated that the phosphorylation of ribosomal S6 protein, which is stimulated by growth factor-binding in a number of cells (Chambard and Pouysségur (1986)), is prevented by conditions which acidify the cell interior (Pouysségur et al. (1982a). Likewise, by using agents such as the diuretic, amiloride and its derivatives which are known to inhibit the exchanger, it has been shown that blocking Na^+/H^+ exchange can also inhibit ribosomal S6 phosphorylation (Pouysségur et al. (1982a)). Moreover, stimulation of DNA synthesis in G_o arrested fibroblasts by growth factors is inhibited by amiloride and its analogues in the same rank order as that for Na^+/H^+ exchanger inhibition (L'Allamain et al. (1984)) and mutants, derived from the fibroblast line CCL39, which lack Na^+/H^+ exchange activity, cannot re-initiate DNA synthesis at extracellular pH values of 7.2 or below (Pouysségur et al. (1985)).

Therefore, several lines of evidence indicate, with relative certainty, that in bicarbonate-free medium the activation of the Na^+/H^+ antiport and subsequent rise in pH_i is essential for growth factor stimulated DNA replication. The only flaw in this conclusion, in terms of applying it in vivo, is that, within an organism, one of the

principal buffers in the fluids bathing cells is bicarbonate (cited in White et al. (1973)). In bicarbonate containing medium, growth factors fail to elevate pH_i and concentrations of amiloride analogues which block more than 95% of exchanger activity in CCL39 cells do not prevent re-initiation of DNA synthesis (L'Allemain et al. (1984)). Moreover, mutants which lack an exchanger grow normally, irrespective of extracellular pH in bicarbonate buffered medium (Pouysségur et al. (1984)). However, this does not completely discount the role of pH_i in mitogenesis, since it has been shown using A431 cells that, in bicarbonate buffers, intracellular pH is 0.1 to 0.15 pH units higher than in bicarbonate-free solutions over the entire pH range tested (Cassel et al. (1985)). Moreover, another mutant cell line which can reinitiate DNA synthesis at lower extracellular pH levels than its parental line, maintains its intracellular pH at suitably elevated levels. (Pouysségur et al. (1985)).

It is reasonable therefore, to conclude that for a cell to ensure progression through the cell cycle, it must generate an intracellular pH above a certain value and perhaps the frequent observation that growth factors stimulate Na^+/H^+ antiport activity, is a way of assuring that this occurs.

1.B.3 PROTEIN PHOSPHORYLATION:

Increases in the phosphorylation of proteins on serine, threonine and tyrosine residues have been reported to occur during the growth response (Kazlauskas and Di Corleto

(1987)). However, it is difficult to make a universal statement about the alterations in protein phosphorylation seen in response to mitogen stimulation because different sets of proteins are phosphorylated in different cell types. Moreover, the phosphorylation patterns produced are usually the result of an interplay between several different Kinases, operating at different times and for different durations throughout the cell cycle. Nevertheless there are a number of proteins, or types of proteins, which are commonly phosphorylated by several different growth factor systems. For example, platelet-derived growth factor (PDGF), bombesin and epidermal growth factor (EGF) all stimulate the phosphorylation of ribosomal S6 protein (Chambard and Pouysségur (1986); Lawen and Martini (1985); Vara and Rozengurt (1985)). Likewise, a number of the cytoskeletal proteins are phosphorylated in response to mitogen stimulation (Sato et al. (1985)) as are the membrane located channels and carriers such as the Na^+/H^+ antiport (Burns and Rozengurt (1983)).

Because alterations in the phosphorylation state of a protein usually results in a modification of its biological activity, the increased phosphorylation patterns may represent a host of secondary cellular changes. Furthermore, because sets of proteins appear to be phosphorylated in co-ordination with one another, assessing the significance of individual protein phosphorylations is ambiguous. Nevertheless, by correlating the onset of these phosphorylation events with DNA replication, good circumstantial evidence has been provided that many of the noted changes in the phosphorylation state represent important

events in regulating the transition of the cell through certain parts of the growth cycle. For example, a study, using quiescent fibroblasts from a number of species (Kohno (1985)), showed that stimulation with a variety of growth factors led to a rapid and marked increase, in the phosphorylation of two proteins, which was half maximal at 5 minutes after exposure to mitogens and diminished gradually after 30 minutes. Mitogen-induced phosphorylation of these proteins was correlated to the extent of mitogen stimulated DNA synthesis and such increases were not observed in exponentially growing cells, suggesting that these phosphorylations could constitute an early event involved in the control of cellular $G_o \rightarrow G_1$ transition. Alternatively, a study in rat liver cells, (Kleine et al. (1986)), arrested in late G_1 phase by incubating the cells in a calcium deficient medium, indicated that lowering of the calcium, even in the presence of serum, not only halted the cell transit through the cell cycle but also, strikingly decreased the phosphorylation of several trypsin-sensitive cell surface proteins. Accordingly, raising the Ca^{2+} concentration stimulated the phosphorylation of certain sets of surface proteins within 5 minutes and led to the initiation of DNA replication within two hours. Therefore, maintenance of these phosphorylation states may be part of the mechanism which triggers the Ca^{2+} dependent $G_1 \rightarrow S$ transition.

1.C SIGNAL TRANSDUCTION MECHANISMS

Once the initial events associated with mitogenesis have been identified, the next step is to determine how the

binding of growth factors to their receptors generates such events. Until relatively recently, little was known about the way external messages are transduced across the membrane. The first success in the search for the intracellular molecules which carry such messages (the so-called second messengers) came with the discovery that intracellular levels of adenosine-3',5'-cyclic monophosphate (c-AMP) were elevated in response to a wide variety of hormones and neurotransmitters. (see Robison et al. (1971)) Since that time an additional second messenger system has been proposed which involves the hydrolysis of a class of membrane phospholipids known as the phosphoinositides (Michell (1975)).

1.C.1 c-AMP SIGNAL TRANSDUCTION SYSTEM:

The mechanics of c-AMP generation are now reasonably well understood (reviewed in Birnbaumer et al. (1985)). Binding of an agonist to its receptor forms a transmembrane complex which acts via a guanine nucleotide binding protein (G protein) known as G_s or N_s to activate the enzyme adenylate cyclase. G_s consists of three subunits, α, β and γ. In the absence of an agonist, guanine diphosphate (GDP) is bound to the α subunit. However the formation of an agonist-receptor complex allows exchange of GDP for guanine triphosphate (GTP) leading to the dissociation of α- from $\beta\gamma$-subunits. It is the free α-GTP which activates adenylate cyclase. Hydrolysis of the bound GTP inactivates the α-subunit thus terminating the signal. Activation of adenylate cyclase promotes the conversion of ATP to c-AMP and pyrophosphate with a subsequent increase in

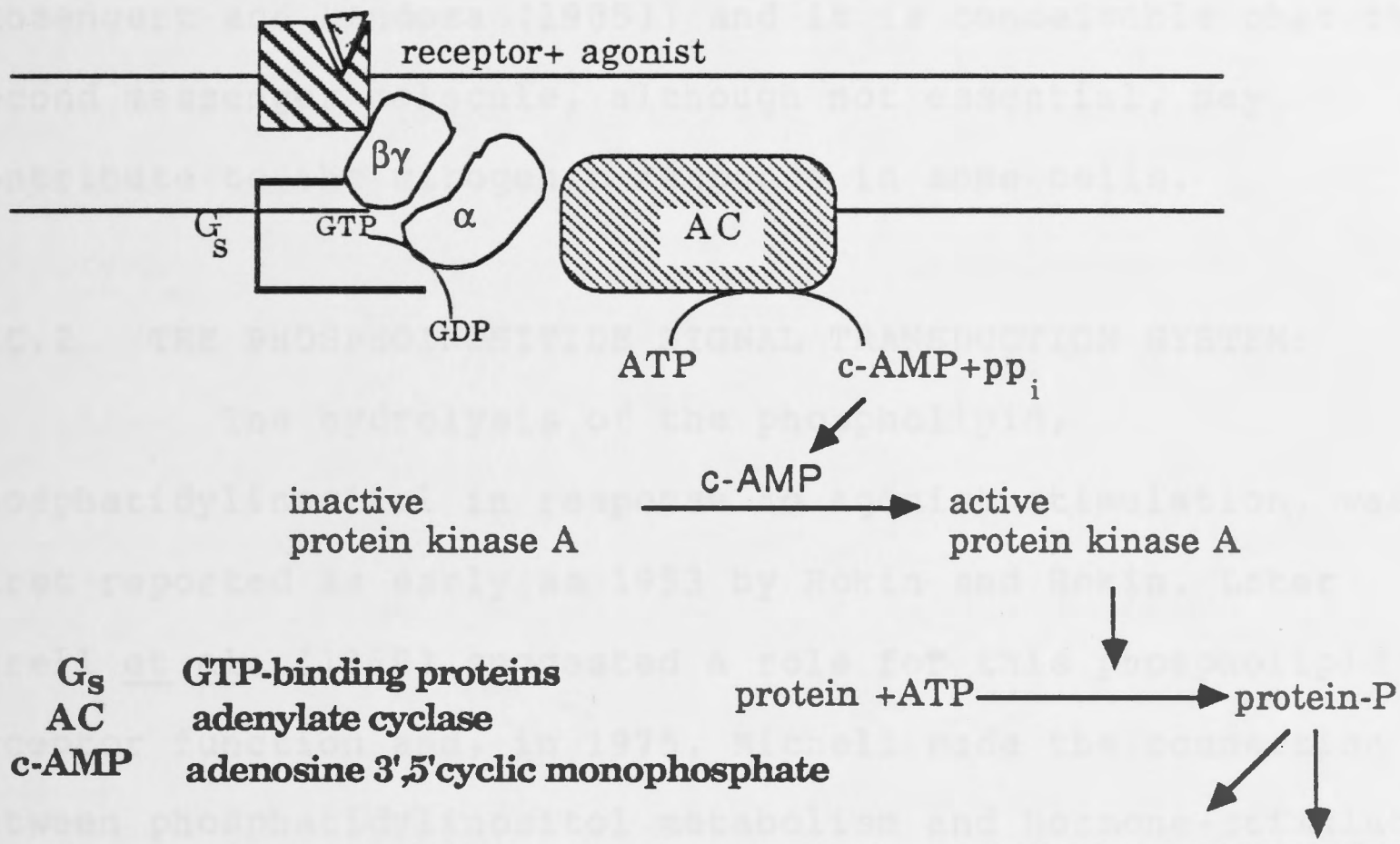
$[c\text{-}AMP]_i$. The c-AMP then interacts with c-AMP-dependent protein kinase (protein kinase A), which in turn phosphorylates specific proteins on threonine and serine residues (see Diagram 1.2). The identity of the target proteins for this kinase vary widely among different cell types and, because many of the target proteins are themselves enzymes, the initial signal can be readily and diversely amplified. Control of this second messenger system occurs by removal of the c-AMP via phosphodiesteric cleavage or through diffusion from the cell (Hunt and Martin (1980)) or, at the level of the receptor, via another G protein, (G_i or N_i). Receptor-mediated dissociation of this protein leads to the release of an inactive α-subunit and a $\beta\gamma$ subunit, the latter of which binds and inactivates the active α-GTP from G_s. (Litosch and Fain (1986)).

Although the c-AMP system most elegantly describes how the extracellular messages of a wide variety of hormones and neurotransmitters can be decoded into something usable within the cell (Robison et al. (1971)), it does not appear to be the means by which the majority of growth factors generate a mitogenic response. Elevation of $[c\text{-}AMP]_i$ fails to generate the monovalent ion fluxes associated with mitogenesis (Rozengurt and Mendoza (1985)) and the activation of the adenylate cyclase cascade can occur without an accompanying rise in $[Ca^{2+}]_i$, characteristic of the response to many growth factors. Moreover, increases in c-AMP have not consistently been correlated with DNA synthesis and, if anything, have more often been associated with having a

DIAGRAM 1.2

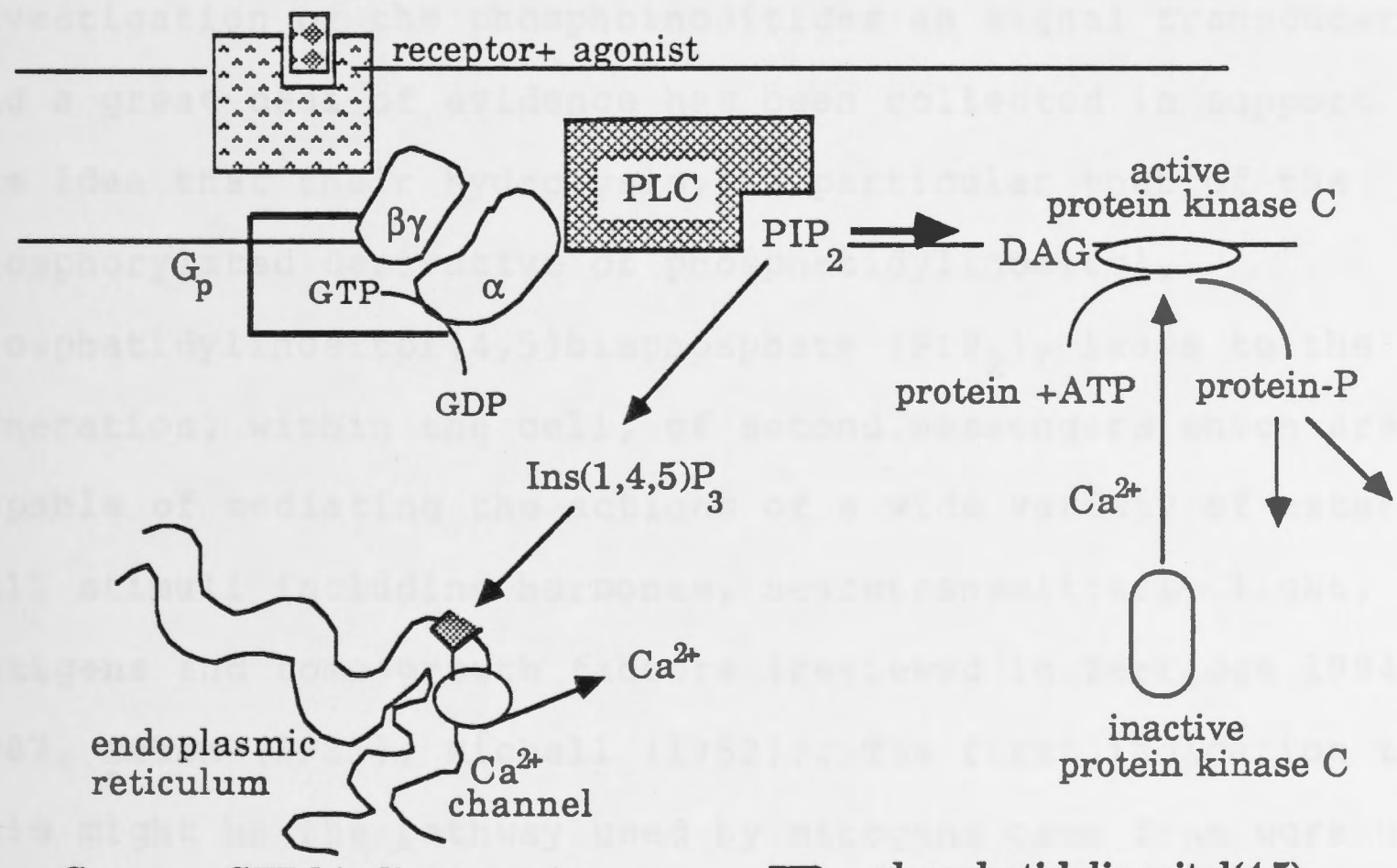
SIGNAL TRANSDUCTION SYSTEMS

ADENYLATE CYCLASE CASCADE



G_s **GTP-binding proteins**
AC **adenylate cyclase**
c-AMP **adenosine 3',5'cyclic monophosphate**

PHOSPHOINOSITIDE PATHWAY



G_p **GTP-binding proteins**
PLC **phospholipase C**
$Ins(1,4,5)P_3$ **Inositol(1,4,5)trisphosphate**
PIP_2 **phosphatidylinositol(4,5)-bisphosphate**
DAG **diacylglycerol**

negative effect on cell proliferation (Hunt and Martin (1980). Nevertheless, sustained elevations of c-AMP have been reported in response to the receptor binding of some growth factors (Rozengurt and Mendoza (1985)) and it is conceivable that this second messenger molecule, although not essential, may contribute to the mitogenic response in some cells.

1.C.2 THE PHOSPHOINOSITIDE SIGNAL TRANSDUCTION SYSTEM:

The hydrolysis of the phospholipid, phosphatidylinositol in response to agonist stimulation, was first reported as early as 1953 by Hokin and Hokin. Later Durell et al. (1969) suggested a role for this phospholipid in receptor function and, in 1975, Michell made the connection between phosphatidylinositol metabolism and hormone-stimulated changes in $[Ca^{2+}]_i$. Since then, there has been much investigation of the phosphoinositides as signal transducers and a great deal of evidence has been collected in support of the idea that their hydrolysis, in particular that of the phosphorylated derivative of phosphatidylinositol, phosphatidylinositol(4,5)bisphosphate (PIP_2), leads to the generation, within the cell, of second messengers which are capable of mediating the actions of a wide variety of external cell stimuli including hormones, neurotransmitters, light, antigens and some growth factors (reviewed in Berridge 1984, 1987, Exton (1985), Michell (1982)). The first indication that this might be the pathway used by mitogens came from work using lymphocytes (Fisher and Mueller (1968)). Since then, growth factors such as PDGF (Berridge et al. (1984)) bombesin (Heslop

et al. (1986) and Conconavalin A (Ku et al. (1981)) have been shown to operate through phosphoinositide hydrolysis. Although much of the information concerning this pathway has been gained by the study of hormone receptor based systems, the fundamental conclusions still apply to the mitogenic response. Because the operation of the phosphonositide pathway forms the basis of the experimental approach to this thesis, the information currently available concerning the operation of this pathway will be considered in some detail.

1.C.2.1 Receptor Mediated Cleavage of Phosphatidyl-inositol (4,5) bisphosphate

The phosphoinositides are a small sub-class of membrane phospholipids, making up less than 8% of the total phospholipid pool (Hawthorne (1982)). They turn over at a higher rate than other phospholipids and, uniquely, the inositol head group of phosphatidylinositol (PI) can be successively phosphorylated to form phosphatidylinositol(4)-phosphate (PIP) and phosphatidylinositol(4,5)bisphosphate (PIP_2) and subsequently dephosphorylated in what is known as the futile cycle (Berridge and Irvine (1984)). PIP_2 appears to be the major precursor used by the receptor mechanism to generate second messengers (Berridge and Irvine (1984)) although there are now suggestions that PIP may also be a target for hydrolysis (Downes and Michell (1982)).

When calcium-mobilizing growth factors bind to their receptors, they activate a phosphoinositide-specific-phospholipase C resulting in the phosphodiesteric cleavage of

PIP_2. This yields two molecules, both of which have been ascribed a second messenger function. These are inositol (1,4,5)trisphosphate ($Ins(1,4,5)P_3$) which, being aqueous soluble, is released into the cytoplasm and 1,2-sn-diacylglycerol (DAG) which remains within the membrane (Berridge (1987)) (see diagram 1.2).

Despite its widespread distribution, phosphoinositide specific-phospholipase C (PLC) has not yet been fully characterised. Purification studies have largely been confined to the cytosolic form which, surprisingly, appears to predominate in most tissues examined (Carter et al. (1986)). Several lines of evidence suggest that the activity ascribed to PLC is frequently derived from multiple forms of the enzyme (Carter et al. (1986), Wilson et al. (1984)). Recently, there have been a growing number of reports of PLC activity in purified plasma membranes (Downes and Michell (1981), Cockcroft et al. (1984)). However, it is not yet clear whether this reflects the ability of the cytosolic form to translocate to the membrane or whether they are two distinct enzymes. Perhaps the two most biologically important characteristics of the enzyme's activity are its calcium dependence and substrate specificity.

1.C.2.1.1 Calcium Dependence of Phospholipase C

Determination of calcium dependence is particularly important due to the proposed role of the enzyme in generating the events which lead to a rise in intracellular free calcium. If phosphoinositide hydrolysis is to be considered as a valid

candidate for the original signal transduction mechanism, then it must be shown to precede the reported calcium rise and in the initial stages, at least, be independent of that rise. Studies examining phosphoinositide hydrolysis in intact cells in the presence or absence of extracellular calcium, or after artifically raising the cytosolic calcium with ionophores, generated mixed conclusions. (Sasaki and Hasegawa-Sasaki (1985); Moscat et al. (1986); Yano et al. (1985)). Studies, using purified PLC to investigate Ca^{2+} sensitivity, were also in disagreement until a thorough study by Irvine et al. (1984) indicated that the in vitro assessment of Ca^{2+} dependence of PLC, depended largely on the assay conditions used. From this and other findings (Downes and Michell (1982); Cockcroft et al. (1984)) it was concluded that in vivo PLC is normally inactive over the entire physiological $[Ca^{2+}]_i$ range.

How, then, does receptor occupation lead to activation of PLC? The Irvine study suggested that PLC could be induced to attack its substrate under in vivo ionic conditions by conversion of its substrate to a non-bilayer configuration and, from this, it was proposed that receptor occupation could led indirectly to PLC activation by generating perturbations in the membrane (Irvine et al. (1984)). However, an alternative explanation which is now gaining a great deal of experimental support, is that receptors are coupled directly to a membrane associated PLC via interaction with one or more distinct GTP binding proteins (designated $G_p/N_p \cong G_s$ and N_i) in a manner analogous to that of the adenylate cyclase system (see diagram 1.2).

Coupling between receptors and PLC was first demonstrated for the blowfly salivary gland where it was shown that 5-hydroxytryptamine could stimulate the enzyme in the presence of GTP (Litosch et al. (1985)). These initial findings have been confirmed in a variety of cell types and the accumulated support for the role of a GTP binding protein in regulating PLC has now been the subject of several reviews (Cockcroft (1987); Smith et al. (1986); Litosch and Fain (1986); Snyderman et al. (1986); Gilman (1987)). With this model for receptor mediated activation of PLC, the controversy over the Ca^{2+} dependence of the phosphoinositide hydrolysis can now be reconciled. It has now been shown that a certain level of Ca^{2+} is, in fact, required for PLC activity. Concentrations of calcium in the 100nM range are essential for optimal activation of the enzyme by both GTP-γ-S (which dissociates the subunits of G_p) and agonists (Uhing et al. (1985)). However, since the $[Ca^{2+}]_i$ of unstimulated cells is around 150nM (Moolenaar et al. (1984)), this requirement can be fulfilled without the need for a rise in cytosolic free calcium. In addition, in some cells, the subsequent rise in $[Ca^{2+}]_i$ to the micromolar range may further promote the hydrolysis of the inositol lipids to maintain the initial rate of hydrolysis (Ciapa and Whitaker (1986)). It is likely that this positive feedback mechanism may prevail in those systems where stimulation of the response is biphasic, an initial component followed by a sustained component. It has also been shown, however, that high levels of Ca^{2+} can activate PLC directly, without operating via the G_p protein (Cockcroft

(1986)), thereby explaining the ability of ionophores to mediate phosphoinositide hydrolysis.

These observations are further confirmed by a recent study (Renard et al. (1987)) which, unlike previous work, monitored $[Ca^{2+}]_i$ in intact cells in relation to agonist-stimulated PLC activity, using a fluourescent Ca^{2+} indicator (QUIN-2). It was shown that PLC activity is little affected by $[Ca^{2+}]_i$ above 193nM but is partially dependent on Ca^{2+} below these levels, lending strong support to the idea that PLC mediated phosphoinositide hydrolysis occurs at basal Ca^{2+} levels.

1.C.2.1.2 Substrate Specificity of Phospholipase C:

Like Ca^{2+} dependency, the substrate specificity of PLC is also important because of the proposed dual nature of the second messengers produced. Although hydrolysis of any of the phosphoinositides would yield DAG, only PIP_2 would produce the triply phosphorylated inositol trisphosphate, which acts to raise $[Ca^{2+}]_i$ from internal stores (see Section 1.C.2.2). The assessment of substrate specificity using endogenous substrates is made difficult by the rapid interconversion, not only between the lipid precursors but also the inositol phosphates produced (Roth (1987)). Studies using purified enzyme from sheep seminal vesicles (Wilson et al. (1984)) have indicated that PLC can hydrolyse all the phosphoinositides, in order of preference PIP_2>PIP>PI and that the phospholipid composition of the membrane can selectively influence PI hydrolysis. However, several studies

using membrane associated PLC activities have indicated that PI is not a substrate for PLC under conditions similar to those *in vivo* (Irvine *et al*. (1984); Uhing *et al*. (1985)). In most cases PIP_2 appears to be the prime target for receptor mediated hydrolysis, although some studies indicate that the relative amounts of PIP and PIP_2 cleaved, depends largely on substrate availability (Downes and Michell (1981)) and perhaps, on the forms of PLC present. In addition it appears that not all membrane PIP_2 is available for PLC attack (Muller *et al*. (1986)).

There is growing evidence in platelets that PLC may be proteolytically activated in response to agonists (Low *et al*. (1984)). However, this phenomenon seems confined to the platelet enzyme which appears to show other unique characteristics (Manne and Kung (1987)) and obviously is not involved in the mitogenic response.

1.C.2.2 Role for Inositol(1,4,5)trisphosphate in raising Intracellular free Calcium

Inositol(1,4,5)trisphosphate is thought to function as a second messenger by liberating Ca^{2+} from non-mitochondrial intracellular stores, thereby mediating the rise in intracellular free calcium seen in response to agonist stimulation. This second messenger role for $Ins(1,4,5)P_3$ was first proposed by Berridge and Irvine (1984) and has since been supported by observations in a variety of cell systems (for review see Berridge (1986); Williamson *et al*. (1985)).

Not only do kinetic studies indicate that the timecourse of $Ins(1,4,5)P_3$ formation matches the agonist

induced $[Ca^{2+}]_i$ rise (Irvine et al. (1985)) but studies using semi-permeablized cells have shown that direct application of $Ins(1,4,5)P_3$ leads to release of Ca^{2+} from internal stores (Muto et al. (1986); Biden et al. (1984); Hirata et al. (1984); Wolf,B.A. et al (1985)). It now appears certain that the principle location of this $Ins(1,4,5)P_3$ sensitive store is the endoplasmic reticulum (E.R.) (Muallem et al (1985); Prentki et al. (1985)) and there is good evidence that $Ins(1,4,5)P_3$ acts through a specific receptor (Spät et al. (1986)). Uptake of Ca^{2+} into the store is ATP dependent and is mediated by the electrogenic Ca^{2+}-ATPase, which requires a compensatory flow of K^+ out of the E.R. to maintain electroneutrality (Muallem et al. (1985)). Release of Ca^{2+} induced by $Ins(1,4,5)P_3$ also requires an opposite flow of the counter ion, K^+, but is ATP independent and is thought to be mediated by activation of a channel rather than inhibition of the uptake system (Adunyah and Dean (1986); Smith et al. (1985)). Calcium release is half maximal between 0.1-3.0μM $Ins(1,4,5)P_3$ depending on the tissue examined (eg see Berridge (1987)) which is in the range estimated in stimulated cells (eg Wollheim and Biden (1986); Aub and Putney (1984)).

Structure-activity studies reveal that the vicinal phosphates on the 4' and 5' positions of the inositol ring are essential for full calcium mobilizing ability and that the affinity of the molecule for its putative receptor is greatly enhanced by having a phosphate on the opposite side of the molecule with a preference for the one position (Burgess et al.

(1984); Irvine et al. (1986)). Although the receptor is specific for Ins(1,4,5)P_3, at higher concentrations, glyceroinositol(4,5)P_2, Ins(2,4,5)P_3 and Ins(4,5)P_2 are also active in mobilizing Ca^{2+} (Putney et al. (1986)). No release occurs upon addition of inositol, Ins(1)P, Ins(2)P, Ins(1:2)cyclic P or Ins(1,4)P_2 (Putney et al. (1986); Burgess et al. (1984)).

Several studies have indicated that not all of the Ca^{2+} stores within the E.R. are Ins(1,4,5)P_3-sensitive (Berridge and Irvine (1984); Muto et al. (1986) but see Prentki et al. (1985)) prompting the suggestion that, in some cells, those sections of the E.R. containing Ins(1,4,5)P_3-gated channels are compartmentalized to localize the response. Nevertheless, in most cells examined Ins(1,4,5)P_3 is capable of releasing more than 50% of stored calcium within seconds of formation which is sufficient to raise $[Ca^{2+}]_i$ to a level where it can generate a physiological response (Brass and Laposata (1987); Biden et al. (1984)).

It has been noted, however, that in many systems, including those involved in the proliferative response, the initial rise in intracellular free calcium, generated by the liberation from internal stores, is usually prolonged by the additional entry of extracellular calcium mediated by activation of channels located in the plasma membrane (Putney (1978); Joseph et al. (1985)). There is some evidence to suggest that inositol(1,4,5)P_3 may also mediate these transmembrane calcium fluxes. A study involving single channel recordings of Ins(1,4,5)P_3-activated conductance in excised

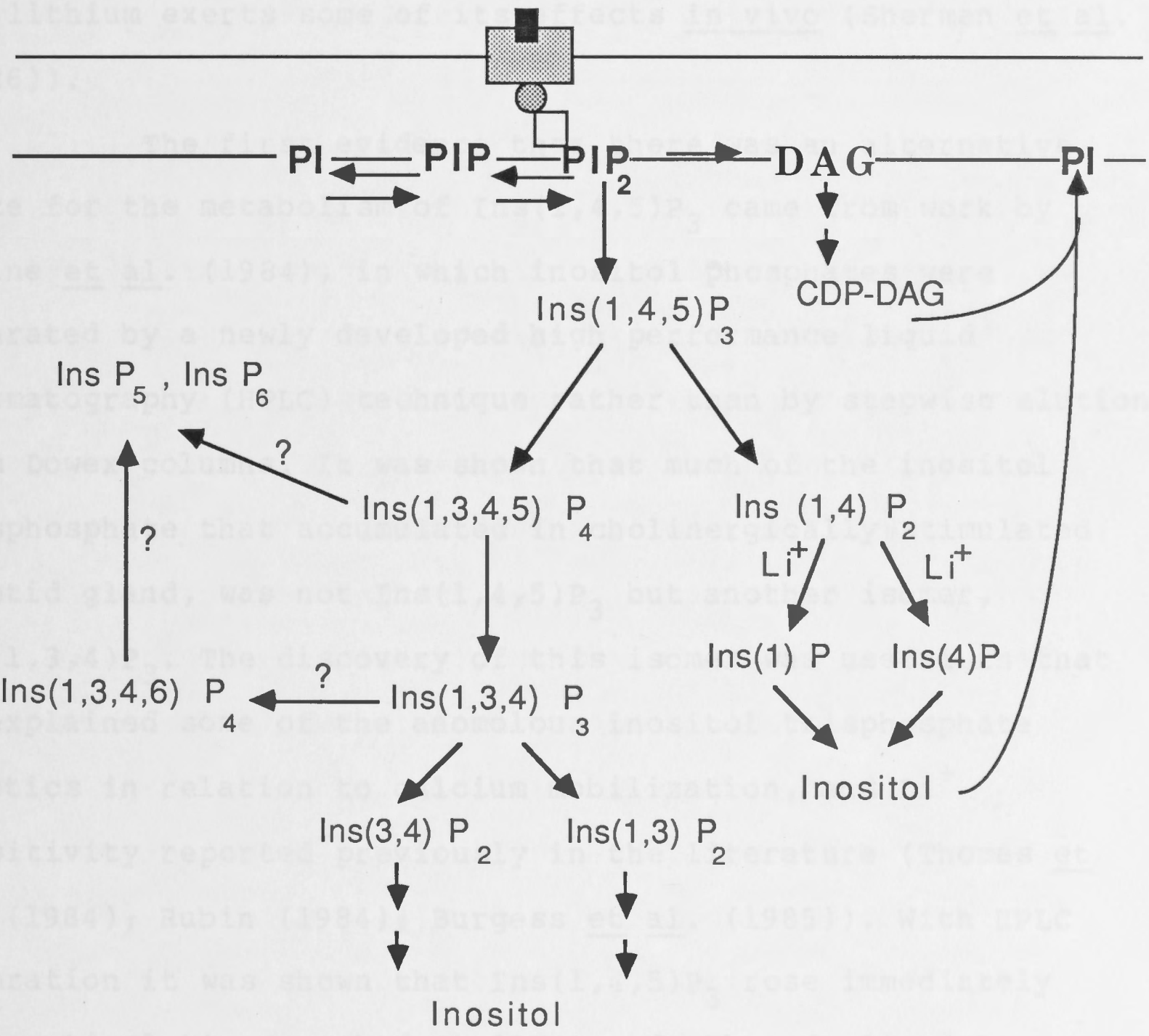
patches of T-lymphocyte plasma membranes (Kuno and Gardner (1987)), has shown that not only could addition of $Ins(1,4,5)P_3$ lead to channel activation but that the $Ins(1,4,5)P_3$-activated channel appeared to be identical to the mitogen activated, voltage-insensitive, calcium permeable channel involved in T cell activation. It has not been verified in these reports, however, whether $Ins(1,4,5)P_3$ is acting directly on the plasma membrane or through a metabolite. (See next Section.)

1.C.2.3 Metabolism of $Ins(1,4,5)P_3$ and Other Inositol Phosphates

In accordance with its role as a second messenger, $Ins(1,4,5)P_3$ is rapidly metabolised (see diagram 1.3). Until recently, it was thought that the only mechanism by which it was inactivated was through the action of a series of phosphatases which successively dephosphorylate $Ins(1,4,5)P_3$ to free inositol (Storey et al. (1984)). The first reaction involves removal of the 5-phosphate to give $Ins(1,4)P_2$ (Downes et al. (1982)), which, as mentioned earlier, is incapable of mobilizing Ca^{2+} from internal stores. $Ins(1,4)P_2$ is then dephosphorylated to either Ins(1)P or Ins(4)P and these subsequent mono-phosphates are then converted to free inositol which may be re-cycled back to phosphatidylinositol (Storey et al. (1984)). This pattern of $Ins(1,4,5)P_3$ degradation has been documented in many cell types and the specific phosphatases mediating these reactions have been characterized (reviewed in Majerus et al. (1988);

DIAGRAM 1.3

METABOLISM OF INOSITOL(1,4,5)TRISPHOSPHATE



Downes (1986)). Lithium ions have been shown to inhibit inositol phosphate metabolism with the primary site of action being inositol(1)monophosphatase (Hallcher and Sherman (1980)) which converts Ins(1)P to inositol. This characteristic has been used experimentally as a method of sensitizing the measurement of agonist induced inositol phosphates by preventing breakdown, (Berridge et al. (1982)) and may explain how lithium exerts some of its effects in vivo (Sherman et al. (1986)).

The first evidence that there was an alternative route for the metabolism of $Ins(1,4,5)P_3$ came from work by Irvine et al. (1984), in which inositol phosphates were separated by a newly developed high performance liquid chromatography (HPLC) technique rather than by stepwise elution from Dowex columns. It was shown that much of the inositol trisphosphate that accumulated in cholinergically stimulated parotid gland, was not $Ins(1,4,5)P_3$ but another isomer, $Ins(1,3,4)P_3$. The discovery of this isomer was useful in that it explained some of the anomolous inositol trisphosphate kinetics in relation to calcium mobilization, and Li^+ sensitivity reported previously in the literature (Thomas et al. (1984); Rubin (1984); Burgess et al. (1985)). With HPLC separation it was shown that $Ins(1,4,5)P_3$ rose immediately after stimulation, peaked at 15 seconds then declined to baseline by 1 minute. $Ins(1,3,4)P_3$ production, on the other hand, did not occur before a 5 second lag and continuously rose over 15 minutes in most cells examined (Burgess et al. (1985), Irvine et al. (1985)).

Because neither the logical lipid precursor nor an isomerase could be found within cells, the origin of $Ins(1,3,4)P_3$ was a mystery until the discovery of yet another agonist-induced inositol phosphate, identified as inositol(1,3,4,5)tetrakisphosphate (Batty et al. (1985)). It now appears certain that this $Ins(1,3,4,5)P_4$ is produced by the phosphorylation of the original messenger $Ins(1,4,5)P_3$ (Hawkins et al. (1986); Stewart et al. (1986)) and then dephosphorylated to $Ins(1,3,4)P_3$ (Downes et al. (1986). The $Ins(1,3,4)P_3$ may then be dephosphorylated to either $Ins(3,4)P_2$ (Dillon et al. (1987)) or $Ins(1,3)P_2$ (Hansen et al. (1986)), the dephosphorylations being lithium-sensitive. In a recent report using angiotensin stimulated adrenal glomerular cells, it has been suggested that $Ins(1,3,4)P_3$ may also be re-phosphorylated to a novel tetrakisphosphate which lacks a phosphate in the 5 position (Balla et al. (1987)). This idea is particularly interesting due to the recent reports of the presence of inositol pentakisphosphate ($InsP_5$) and inositol hexakisphosphate ($InsP_6$) within mammalian cells (Vallejo et al. (1987)).

$Ins(1,3,4,5)P_4$ levels peak 20 seconds after stimulation (Hawkins et al. (1986)) and because of its rapid formation and hydrolysis, a second messenger role for this molecule has been sought. It has been shown in a number of cell types that, unlike $Ins(1,4,5)P_3$, $Ins(1,3,4,5)P_4$ is incapable of mobilizing calcium from internal stores (Irvine and Moor (1986)). It was thought, instead, that it may mediate the subsequent entry of extracellular calcium, seen as a

secondary response in most cells after agonist stimulation. This idea was tested by Irvine and Moor (1986), by microinjection of submicromolar concentrations of $Ins(1,3,4,5)P_4$ into sea urchin eggs. It was found that this caused a raising of the fertilization envelope (egg activation) and that this effect was dependent on both the presence of extracellular calcium and on the co-injection of a Ca^{2+} mobilizing compound such as $Ins(2,4,5)P_3$. The provision that extracellular calcium be present indicated that $Ins(1,3,4,5)P_4$ is probably activating a calcium entry system located in the plasma membrane and, in fact, specific receptors for $Ins(1,3,4,5)P_4$ have been reported on membranes of HL-60 cells (Bradford and Irvine (1987)). The requirement for the co-injection of an agent capable of liberating Ca^{2+} from internal stores is more intriguing. It was suggested that perhaps this requirement was peculiar to the egg system but it has since been shown in lacrimal acinar cells that $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ act in concert (but not individually) to activate Ca^{2+} - dependent K^+ channels leading to an influx in Ca^{2+} (Morris et al. (1987)). This may suggest that $[Ca^{2+}]_i$ must already be elevated for $Ins(1,3,4,5)P_4$ to be effective. Alternatively, as proposed by Putney (1986), it may be that the $Ins(1,4,5)P_3$ mobilizable intracellular calcium stores must be empty before extra-cellular calcium can be recruited. Whatever the mechanism (Irvine (1987)), the ability of $Ins(1,3,4,5)P_4$ to stimulate entry of Ca^{2+} into the cell is interesting, considering that the kinase which converts $Ins(1,4,5)P_3$ to $Ins(1,3,4,5)P_4$ is

calcium sensitive, being regulated by calmodulin (Biden *et al.* (1987)). Therefore, the initial liberation of Ca^{2+} from intracellular stores not only promotes the metabolism of the effector but produces a compound capable of prolonging the response in what appears to be a self-potentiating system.

As yet, no second messenger function has been ascribed for $Ins(1,3,4)P_3$ and it has been proposed that the kinetics of its formation may argue against it playing an active part in a signal transduction event. Although it has been reported to mobilize intracellular calcium in several cell types, its potency in most cases is much lower than that of $Ins(1,4,5)P_3$ (Irvine *et al.* (1986)) and it is generally considered that catabolism to $Ins(1,3,4)P_3$ is the means by which the calcium mobilization signal, initiated by $Ins(1,4,5)P_3$, is terminated. However, $Ins(1,3,4)P_3$ may have some, as yet, unidentified signal transducing ability, either in its own right or as a potential precursor for the biologically active polyphosphates (IP_5 and IP_6).

Yet another group of inositol phosphates which may be involved in signal transduction, are the cyclic inositol phosphates. It has been known for some time that the action of phosphoinositide-specific phospholipase C on phosphatidylinositol *in vitro*, leads to generation of Ins(1:2) cyclic-monophosphate (Dawson and Clarke (1972)) as well as the non-cyclic derivative (Ins(1)P) and it has now been shown that the cleavage of PIP_2 and PIP by purified PLC from seminal vesicles also generates cyclic compounds (Wilson *et al.* (1985)). Moreover, although the ability of $cIns(1,2:4,5)P_3$

to mobilize intracellular calcium varies with the tissue examined it has been found to be as potent as Ins(1,4,5)P_3 in some cell types (Wilson et al. (1985a)).

Until recently it was not clear whether cyclic inositol phosphates were produced in stimulated cells because these compounds did not survive the acidity of the extraction conditions employed. However, it has now been shown that cyclic inositol phosphates are generated in response to agonist stimulation in a number of cell types (see Majerus et al. (1986)). It seems likely that the cyclic phosphates will be degraded, albeit more slowly, by the same phosphatases which degrade Ins(1,4,5)P_3 with the ring being opened only after dephosphorylation to cIns(1:2)P (Majerus et al. (1986)). The enzyme capable of cleaving this compound has been isolated and characterized (Ross and Majerus (1986)). In addition, isolation of two isozymes of phospholipase C from lymphocytes has indicated that, with phosphatidylinositol as a substrate, distinct ratios of Ins(1)P to cIns(1:2)P are formed with the different enzymes, suggesting a potential mechanism, operating at the level of the receptor, whereby the relative amounts of the cyclic inositol phosphates can be regulated in response to agonist stimulation (Carter et al. (1986)).

1.C.2.4 Diacylglycerol Activation of Protein Kinase C

The principle means by which diacylglycerol (DAG) is thought to act as a second messenger within the cell is through its ability to directly activate the enzyme, protein kinase C (Takai et al. (1979)). It now appears, however, that the

metabolism of DAG, like that of Ins(1,4,5)P_3, may also generate further biological regulators. (See Section 1.C.2.4.8)

1.C.2.4.1 Properties of Protein Kinase C:

Protein kinase C (PKC) was originally described in 1977 as a proteolytically activated proenzyme (Inoue et al. (1977)). However, it has since been found that it may be reversibly activated by association with membrane phospholipids in the presence of physiological Ca^{2+} concentrations and DAG, (Takai et al. (1979); Kishimoto et al. (1980)) leading to the name Ca^{2+}-activated and phospholipid-dependent protein kinase. The enzyme is ubiquitously distributed in tissues and organs and it has been purified to homogeneity from many sources. (For reviews see Kikkawa and Nishizuka (1986); Schwantke et al. (1985); Nishizuka (1984)). PKC is composed in all cases of a single polypeptide chain with no sub-unit structure. Neither calmodulin nor any antibody against calmodulin affects the enzyme activity (Kikkawa and Nishizuka (1986)). Magnesium is essential for catalytic activity, the optimal range being about 5-10mM and the optimum pH range for activity (as assessed in Tris acetate buffer) is 7.5-8.0 (Kikkawa and Nishizuka (1986)).

It has been reported that both the kinetics and catalytic activity, of PKC, are almost indistinguishable between the various isolates (Kikkawa and Nishizuka (1986)). However, the molecular weight of the enzyme varies with the technique used and the tissue examined (range 77-84 000M_r as judged by SDS-PAGE). This tissue specific variation was also

noted for other physical properties such as pH optimum and Stokes radius (see Schwantke et al. (1985)), but until recently, it was only speculated that these differences might reflect distinct molecular species. However, with the application of molecular biology technology to the study of PKC, it has been found that there are at least four different forms of the enzyme produced by cells. (For reviews see Parker and Ullrich (1987); Carpenter et al. (1987))

1.C.2.4.2 A Family of Protein Kinase C Genes:

Because several reports of the existence of multiple cDNA species of PKC appeared in the literature within months of one another, initially there were problems with conflicting nomenclature (Coussens et al. (1986) and Ohno et al. (1987): for resolution, see Kubo et al. (1987)). However, the nomenclature used by Coussens et al. (1986) appears to be the one that has now been universally adopted (Carpenter et al. (1987)). In that study, it was shown that three highly related but non-identical PKC sequences could be isolated from both bovine and human brain cDNA libraries, and that the corresponding human genes, designated α, β and γ were localized on to separate chromosomes. At the same time Knopf et al. (1986) had isolated three distinct cDNA clones from rat brain. However, it was later shown that two of these clones, now termed β I and β II, were derived from alternative splicing of the one gene. Ohno et al. (1987), who originally showed the presence of the two splicing alternatives, have recently identified the presence of cDNA clones encoding the four types of PKC (α, β I, β II, γ) in rabbit brain, and work by the same

group has demonstrated the presence of both I and II in a human splenic cDNA library (Kubo et al. (1987)). From this, it was concluded that the presence of all four types of PKC is a common feature of all mammalian cells. In addition, Northern and Southern hybridization analysis suggests the potential for even greater genetic complexity within the PKC gene family (Coussens et al. (1986)).

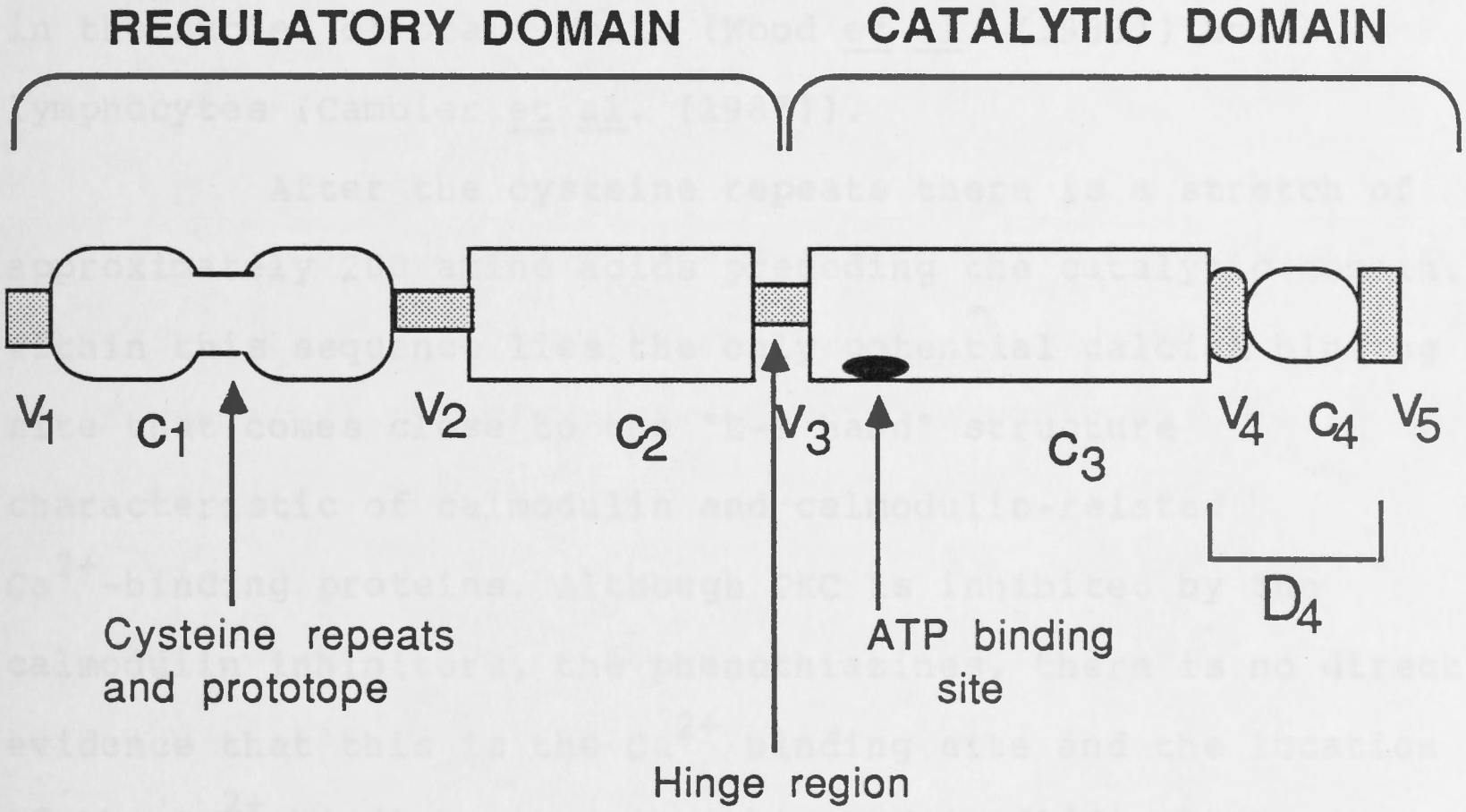
The first sequence-structure analysis was performed on bovine PKC α by Parker et al. (1986). The cDNA selected for analysis was translated into a 76 kDa product with a two-domain structure which could be functionally divided into a catalytic (carboxy terminal) and regulatory (amino terminal) domain (see diagram 1.4). As mentioned earlier, limited proteolysis of PKC leads to the generation of an active fragment that is no longer subject to Ca^{2+}, phospholipid and DAG dependence (protein kinase M) (Kishimoto et al. (1983)).

Using antipeptide antisera, the upper limit of this catalytically active fragment has been defined to the C-terminal 380 residues. Within this C-terminal domain, is a Gly x Gly x x Gly sequence (where X is any other amino acid) which, alongside lysine 368, forms part of the predicted nucleotide (ATP) binding site. In addition, there are several other characteristic stretches, all of which remain highly conserved in all the serine, threonine and tyrosine specific protein kinases.

Towards the amino terminus of the regulatory domain, there is a region that is characterised by a tandem repeat, each repeat unit containing six cysteine residues. Such

Figure 1.4

Domain Srtucture of Protein Kinase C and Regions of Variability between isozymes



cysteine-rich duplication sequences are found in a number of polypeptides, although for the most part, the functional significance of this protein architecture is not clear. It has some similarities to a number of DNA binding proteins where such structures have been proposed to form cysteine (zinc) "fingers" and divalent metal ion co-ordination complexes (Berg (1986)). There is no direct evidence that PKC is a DNA binding protein but PKC activity and immunoreactivity has been reported in the nuclei of brain cells (Wood et al. (1986)) and B lymphocytes (Cambier et al. (1987)).

After the cysteine repeats there is a stretch of approximately 200 amino acids preceding the catalytic domain. Within this sequence lies the only potential calcium binding site that comes close to the "E-F hand" structure characteristic of calmodulin and calmodulin-related Ca^{2+}-binding proteins. Although PKC is inhibited by the calmodulin inhibitors, the phenothiazines, there is no direct evidence that this is the Ca^{2+} binding site and the location of the Ca^{2+}-binding site(s) within PKC is difficult to predict from the primary sequence. There is direct evidence that the diacylglycerol and phospholipid binding sites are located within this regulatory domain but there is no single hydrophobic stretch which might form a suitable site for interaction with membranes. There are predicted amphipathic helices that, as in phospholipase A_2, could provide a site for interaction with the membrane (Maraganore (1987)) but a detailed understanding of the phospholipid/diacylglycerol/ PKC interaction will ultimately require the resolution of its tertiary structure.

A recent study by House and Kemp (1987) has indicated that the regulatory domain of PKC also contains an amino acid sequence between residues 19 and 36 that resembles a substrate phosphorylation site in its distribution of basic residue recognition determinants. This sequence is not a site of autophosphorylation (Huang et al. (1986)) but has the secondary structural features of a pseudosubstrate, termed "prototope" and may be responsible for maintaining the enzyme in the inactive form in the absence of allosteric activators such as phospholipid (House and Kemp (1987)).

When the amino acid sequences of the different PKC types from different species were examined, two interesting points emerged. The first was that, when human and bovine gene products were compared, there was extensive sequence conservation (98%) within all regions of α, β II and γ proteins (Coussens et al. (1986)) and that a comparison of β I sequences from human, rat, rabbit and bovine sources also showed remarkable sequence homology (Kubo et al. (1987)). This interspecies conservation strongly suggests that any variable domains between the different types of PKC must play an important role in defining distinct functional specificities of the proteins rather than functioning as mere structural (or evolutionary) spacers. The second point, therefore, concerns the location of the regions of variability between the types of PKC in relation to their predicted function with the protein (see diagram 1.4).

Alignment of bovine PKC α, β II and γ sequences revealed an overall sequence homology in two major domains that

are separated by one large variable region (V3) (Coussens et al. (1986); Parker and Ullrich (1987)). Overall, PKC γ exhibits the highest degree of divergence within five significantly variable regions designated V_1 to V_5. V_1 and V_2 are either side of the cysteine-rich C_1 region which is highly conserved in all three types of PKC. The proposed pseudosubstrate also lies within this C_1 region. The C_2 region that follows V_2 is 115 amino acids long and may contain a Ca^{2+}-binding site distinct from the 'E-F hand' type. This zone is followed by the most highly variable region V_3. Just within the V_3 region lies the sequence that Parker et al. (1986) originally described as a potential calmodulin-like Ca^{2+} binding site. It is interesting, therefore, that this site is not conserved in either β or γ. If this site is used to bind Ca^{2+} in the αform, the divergence may represent a functional distinction within the PKC family.

Adjacent to this site is the largest region of extensive sequence divergence, which has hydrophilic characteristics in all three PKC sequences. It is likely to represent an exposed surface domain since it is sensitive to proteolytic attack and is proposed to be the 'hinge' region between the regulatory and catalytic domain (Coussens et al. (1986)). Beyond the V_3 region is a highly conserved region that includes a Gly x Gly x x Gly sequence for ATP binding and marks the beginning of the kinase domain. In PKCγ, however, there is a minor insertion of five residues at the carboxyl end of this zone which constitutes V_4. Interestingly, a second Gly x Gly x x Gly consensus sequence for ATP binding sites is

found in all three PKC sequences at the end of the kinase domain, with lysine residues found an appropriate distance downstream in α and β II only. Whether or not these sequences represent an additional ATP binding site requires further investigation. Another minor divergent structural feature is found at the carboxyl terminus (V_5). The α and γ sequences are 2 and 11 residues longer respectively, than the aligned β II sequence.

In a study, by Ohno et al. (1987), which compared the sequence from rabbit β I, β II and α PKC, a similar conclusion concerning the location of the variable region was made. The only deviation was in the assessment of the catalytic domain, whereby, in the latter study, V_4 was not present (because γ was not examined) and the V_5 region (termed D_4 in that study) was extended back to include the last 50 to 55 amino acids. This D_4 zone may be important as it is the only area of difference between the β I and β II gene products from human, rat and rabbit sources. It has been suggested (Ohno et al. (1987)) that D_4 could be involved in the recognition of target proteins in a manner analogous to other systems, suggesting that different PKC types have different substrate specificities. However, there is as yet, no experimental evidence for this.

Three distinct forms of PKC have been isolated from rat brain cytosol by hydroxylapatite column chromatography (Huang et al. (1986)), and, although all three types had many properties in common, including a kinase activity dependent on Ca^{2+} and phospholipid, they could be distinguished from one

another on the basis of either immunoreactivity or autophosphorylation sites. In addition, several studies concerning tissue distribution of m-RNA for PKCα, β I, β II and γ have been performed (Brandt et al. (1987); Ohno et al. (1987)) and it has been clearly shown that different tissues do have different patterns of expression of the varius PKC forms. This information strongly suggests that the different types of PKC have different physiological roles within the cell. This would provide at least part of the explanation as to how the wide variety of hormones, neurotransmitters and growth factors can act through the one phosphoinositide signal transduction pathway and elicit such different responses.

Less than two years ago it was assumed that PKC was a single entity. All the information that has been collected concerning the activation and subsequent role of this enzyme activity in generating a cellular response, has been interpreted in the light of this misconception. The fact that, until recently, there was no need to propose multiple forms of the enzyme to explain experimental data, suggests that the variations between the PKC isozymes may lead to subtle changes in the regulation of enzyme activity rather than overt changes in its character. It is hoped, therefore, that the following sections concerning the means and ramifications of the activation of PKC by DAG are generally valid and that future information gained from studying and distinguishing α, β I, β II and γ PKC, serves only to qualify rather than re-appraise our current knowledge.

1.C.2.4.3 Biochemical and Physiological Activation of Protein Kinase C:

Protein Kinase C, per se, is normally inactive, and in an unstimulated cell is found, in most cases, in the cytosol (Kikkawa and Nishizuka (1986)). However, upon cell stimulation, the enzyme apparently translocates to the membrane where its activity is modulated by membrane components (Takai et al. (1979); Bell (1986)).

When assayed in a cell-free system, protein kinase C (PKC) activation depends totally on phospholipid and unphysiologically high levels of Ca^{2+} (Takai et al. (1979)). However, in the presence of diacyglycerol (DAG), the affinity of the enzyme for Ca^{2+} is greatly increased such that PKC can be fully active at physiological calcium concentrations (Takai et al. (1979a). It is generally considered that under such conditions the only phospholipid capable of activating PKC is phosphatidylserine, the affinity of the enzyme for which is also increased by DAG (Takai et al. (1979a)). However, recent evidence suggests that different substrates for the enzyme generate different lipid requirements (Bazzi and Nelsestuen (1987)). Various sonicated phospholipids have been reported to potentiate or inhibit phosphatidylserine-dependent activation (Kaibuchi et al. (1981)), but this has not been found when mixed micelles or defined lipid vesicles are used and under such experimental conditions, at physiological Ca^{2+} concentrations, the activity of PKC shows close to absolute dependence on DAG (Hannun et al. (1985).

Various DAG are capable of activating the enzyme, (Boni and Rando (1985); Lapetina et al. (1985)) but in

physiological processes 1,stearoyl-2-arachidonyl glycerol is the likely activator since most of the inositol lipids contain this DAG backbone (Holub et al. (1970)). Specificity tests have shown that the active DAG must be of the 1,2-sn configuration, suggesting that a highly specific lipid-protein interaction is needed for enzyme activation (Boni and Rando (1985); Ganong et al. (1986)). This also precludes a role for the DAG produced from triglycerides, by lipoprotein lipase or heparin-releasable hepatic lipase in activation of protein kinase C, as they have a 2,3-sn configuration (Morley and Kuksis (1972); Akesson et al. (1976). The 3-OH group and 1-2 carbonyls are both required, as is a long chain fatty acid, in at least one of the two positions with activity increasing from 3 to 11 carbons (Ganong et al. (1986); Hannun et al. (1985)).

Due to the highly co-operative nature of phosphatidylserine (PS) activation, four or more PS molecules are required per molecule of a enzyme (Hannun et al. (1985); Bell (1986)). Activation by DAG, however, is non-cooperative, requiring only a single molecule. Studies have shown that protein kinase C is fully activated by mixed micelles containing molar fractions of DAG (2.5%) and PS (8%) comparable to those in the plasma membrane during activation of phosphoinositide hydrolysis (Bell (1986)).

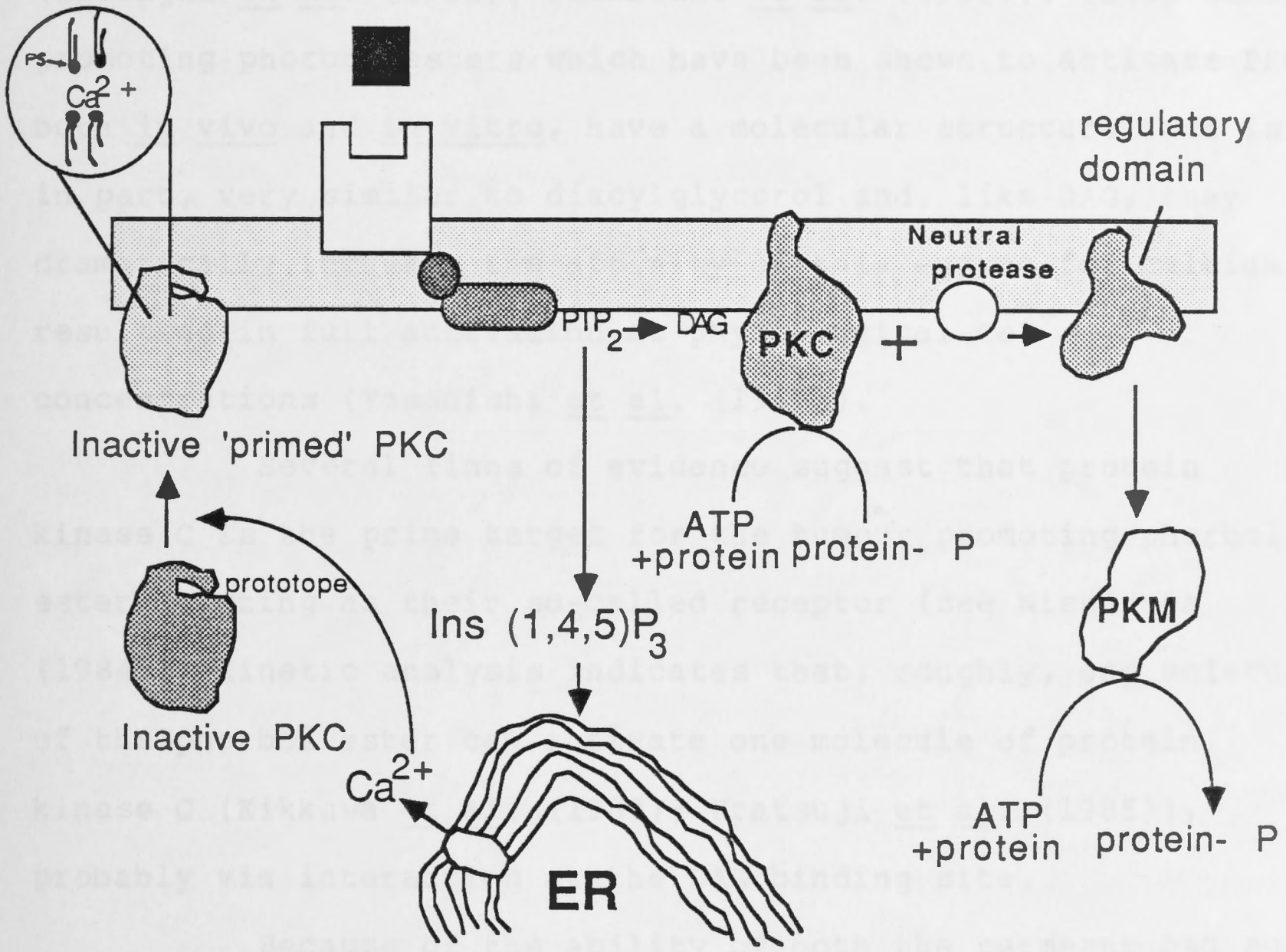
Protein Kinase C can also be activated irreversibly by limited proteolysis, generating a 50.000M_r kinase known as protein kinase M which is both Ca^{2+}- and phospholipid-independent (Inoue et al. (1977); Kishimoto et al. (1983)). Such an activation has been shown to be mediated in vitro by a

Ca^{2+}-dependent neutral proteinase present in cellular membrane fractions (Kishimoto et al. (1983); Melloni et al. (1985); Mizuta et al. (1985)) and is thought to occur in vivo, resulting in the release of the constitutively active PKM into the cytosol, (and presumably leaving the hydrophobic regulatory unit within the membrane) (Melloni et al. (1985)). Enzyme activities attributable to PKM have been reported in the membrane and/or cytosol of keratinocytes (Chida et al. (1986)), platelets (Tapley and Murray (1985)) neutrophils (Melloni et al. (1985a)) and breast cancer cells (Fabbro et al. (1986)) treated with phorbol esters and in B lymphocytes treated with either phorbol ester and calcium ionophore or with the mitogen, SAC (Guy et al. (1986)). The physiological significance of this cleavage is still being debated (Murray et al. (1987); Buday et al. (1987)). However, there is growing evidence that the generation of PKM is not merely a means of degrading PKC (see Murray et al. (1987)). Furthermore, it is noteworthy that the greatest region of sequence variation, between the different forms of PKC, lies within the hinge region containing the predicted site of proteolytic attack (Coussens et al. (1986)). It could be predicted that the different types of PKC have different capacities to be converted to the PKM form, some remaining predominantly within the membrane after activation, others moving to the membrane only to be cleaved. This idea is supported indirectly by studies, examining the phorbol ester-mediated cleavage of PKC, which have shown that the time required for a 50% decrease in PKC activity in keratinocytes was one-sixth to one-seventh the time typical of BALB/3T3 or C3H/10T1/2 cells (Chida et al. (1986)).

Taking this information together, models have been developed (Bell (1986) with Murray et al. (1987)) to explain what may be happening in vivo when protein kinase C is activated during phosphoinositide mediated signal transduction (see diagram 1.5). The first step involves translocation of the inactive cytosolic protein kinase C to the membrane. Studies using inside-out erythrocyte membranes (Wolf et al. (1985a)) have shown that, at Mg^{2+} concentrations similar to those inside the cell, a rise in the intracellular Ca^{2+} concentration, from a basal level (150nM) to that seen after stimulation (1μM), leads to rapid association of protein kinase C with the membrane. This association is promoted by DAG, probably by stabilization of the complex within the membrane. It is suggested that, initially, four molecules of PS form a structure on the surface of the membrane and bind Ca^{2+} through their carboxyl group (Bell (1986)). Protein kinase C binds to this complex of PS and Ca^{2+} in a 'primed' but inactive state. DAG then interacts with the complex at three points (one at Ca^{2+} and two at protein kinase C) which may affect enzyme conformation thereby leading to activation (possibly by displacing the pseudosubstrate from the active site (House and Kemp (1987)) protein Kinase C might then be inactivated by dissociation of DAG from the complex after which it could remain in its primed state in the membrane until $[Ca^{2+}]_i$ decreases. Alternatively, the membrane bound enzyme may be cleaved by the neutral proteinase releasing the de-regulated protein kinase M into the cytosol (Murray et al. (1987)).

Figure 1.5

Model of Protein Kinase C Activation in Intact Cells



1.C.2.4.4 Activators and Inhibitors of Protein Kinase C:

In addition to the endogenously produced DAG, a number of other compounds have been shown to activate PKC, the most notable of which are the tumour promoting phorbol esters (Castagna et al. (1982); Yamanishi et al. (1983)). These tumour promoting phorbol esters which have been shown to activate PKC both in vivo and in vitro, have a molecular structure that is, in part, very similar to diacylglycerol and, like DAG, they dramatically increase the affinity of this enzyme for calcium, resulting in full activation at physiological Ca^{2+} concentrations (Yamanishi et al. (1983)).

Several lines of evidence suggest that protein kinase C is the prime target for the tumour promoting phorbol esters, acting as their so-called receptor (see Nishizuka (1984)). Kinetic analysis indicates that, roughly, one molecule of the phorbol ester can activate one molecule of protein kinase C (Kikkawa et al. (1983); Uratsuji et al. (1985)), probably via interaction at the DAG binding site.

Because of the ability of both the permeant DAG such as 1-oleoyl-2-acetylglycerol (OAG) and the tumour promoting phorbol esters to activate protein kinase C in vivo, they have been used in many systems as experimental tools to examine the role of activated protein kinase C. However, several studies have suggested that the manner in which phorbol esters influence protein kinase C may not be identical to that of DAG or OAG, (Ashby et al. (1985); Bijleveld et al. (1988)) casting doubt on the unqualified suitability of these tumour promotors for cell biology experiments. This may be due, in part, to the

different rate of metabolism of the phorbol esters. DAG is present only transiently in membranes whereas the phorbol esters are metabolised very slowly and persist for longer periods within the membrane (Kikkawa and Nishizuka (1986)). The concentration of phorbol ester used is also an important consideration since at higher concentrations these agents can act as membrane perturbers or fusigens, influencing the cell directly rather than through protein kinase C (cited in Kikkawa and Nishizuka (1986)). However, other structurally unrelated tumour promotors such as mezerein, teleocidin and Aplysia toxin have also been shown to activate protein kinase C (Couturier et al. (1984); Fujiki et al. (1984) further supporting the key role for this enzyme in the regulation of cell growth.

A number of endogenously produced fatty acids and their oxygenated products have also been shown to activate PKC in vitro including arachidonate, lipoxin A and the octa decadienoic acids, linoleic acid and linolelaidic acid (Seifert et al. (1987); Hansson et al. (1986)). Interestingly, it has been reported that the substrate specificity of PKC activated by lipoxin A is different from that of phosphatidylserine or phorbol ester (Hansson et al. (1986)). Moreover, short chain phosphatidylcholines, unlike their long chain counterparts are also capable of activating C-kinase (Walker and Sando (1988)).

Many compounds have been found to inhibit protein kinase C activity but, unfortunately, none are specific for the enzyme. The majority appear to act by disrupting membrane phospholipids (Kikkawa and Nishizuka (1986)). These include a number of the psychotic drugs such as chlorpromazine, local

anaesthetics (e.g. dibucaine), staurosporine, (Tamaoki et al. (1986)) triphenylethylenes, (O'Brian et al. (1986)) anti-calmodulins, polyamines and polymixin B (reviewed in Hidaka and Hagiwara (1987)). These phospholipid interacting drugs usually inhibit calmodulin dependent protein kinases such as myosin light chain kinase. Some compounds, namely the isoquinoline-sulfonamides, have been found which interact with the catalytic site of protein kinase C (Hidaka et al. (1984); Fujita et al. (1986)) but even H7, the most selective of these, also inhibits the activity of the cyclic nucleotide dependent protein kinases to some extent.

Recently several endogenous inhibitors of protein kinase C have been reported which may prove to have both physiological and pharmacological significance. Two Ca^{2+}-binding proteins, of molecular weight 17k Da and 12kDa, have been isolated from bovine brain cytosol, both of which have been shown to inhibit protein kinase C, in a manner which is due neither to interaction with substrates or co-factor nor to phosphatase activity (McDonald and Walsh (1986)). Both also display a certain degree of specificity in that they have no effect on calmodulin-dependent cyclic nucleotide phosphodiesterase or c-AMP-dependent protein kinase and, in the case of the 17kDa Ca^{2+} binding protein, no effect on myosin light chain kinase. A third 40 kDa, heat-stable inhibitor protein with a dimer configuration has also been isolated from bovine brain cytosol but its mode of action and specificity have not yet been reported (Hucho et al. (1987)). In addition, preliminary characterization of an endogenous protein kinase C

inhibitor has been reported in human neutrophils. It is primarily associated with neutrophil-specific grannuole membranes, is protease and heat sensitive and has neither protease nor phosphatase activity (Balazovich et al. (1986)). Alternatively, alkylacylglycerols, produced when choline phospholipids are degraded by phospholipase C, have also been shown to inhibit PKC in a concentration dependent manner. (Daniel et al. (1988)).

1.C.2.4.5 Catalytic Activity and Target Proteins of Protein Kinase C:

Protein kinase C, like c-AMP-dependent protein kinase (PKA), phosphorylates proteins on serine and threonine, but not tyrosine residues in proteins. It uses ATP but not GTP as its phosphate donor (Kikkawa and Nishizuka (1986)). An extensive study using synthetic peptides (House et al. (1987)) has indicated, that unlike PKA, the phosphorylation recognition site used by PKC cannot be described by a simple amino acid sequence. A single basic residue, preferably arginine, on either side of the residue to be phosphorylated, is the primary determinant but additional basic residues and other structural features also influence the kinetics of peptide phosphorylation. The substrate requirements for PKA are more restricted in that there is a specific preference for the arginine residue on the amino side of the phosphate acceptor (Krebs and Beavo (1979)). However, the overlap in site requirements of these kinases may justify the report that, in some instances, PKC and PKA, not only phosphorylate the same

proteins, but also the same residues (Kishimoto et al. (1985)), explaining why such seemingly divergent signal transduction pathways can have certain cellular responses in common. Nevertheless, comparative studies concerning the structural requirements of the two enzymes have indicated that, despite apparent similarities, the primary structural determinants for PKC and PKA are, in general, quite distinct. (Ferrari et al. (1985)).

PKC mediates its positive effects on cell growth by altering the activities of a variety of proteins through phosphorylation and perhaps by acting directly on the genome. In most tissues, conclusive information concerning its in vivo targets is not available. However, the enzyme has been shown to phosphorylate a wide range of proteins in vitro, many of which are phosphorylated in intact cells after treatment with tumour promoting phorbol esters or OAG (Kikkawa and Nishizuka (1986)). Although the physiological relevance of such phosphorylations is not always clear, many of the target proteins obviously have key roles in the growth response.

Phosphorylation and subsequent activation of the Na^+/H^+ antiporter by PKC (Moolenaar et al. (1984a)) not only leads to an intracellular alkalinization, but also stimulates the Na^+/K^+ ATPase, explaining how activators of C-kinase activity have been shown to elicit many of the early ionic events associated with mitogenesis in quiescent 3T3 fibroblasts (Vara et al. (1985)). The rise in intracellular free Ca^{2+}, generated by the $Ins(1,4,5)P_3$ arm of the pathway, can also be modulated by PKC via the kinase's ability

to phosphorylate phospholamban (Movsesian et al. (1984); Lagast et al. (1984)). This results in an increase in the activity of the Ca^{2+}ATPase in the sarcoplasmic (and presumably endoplasmic) reticulum, therefore replenishing the $Ins(1,4,5)P_3$ sensitive calcium stores. Moreover, the ubiquitous phosphorylation of ribosomal S6 protein in response to mitogenic stimulation can result from PKC activation (Parker et al. (1985)) and the alterations in the cytoskeleton, documented to occur during the proliferative response (Herman and Pledger (1985)), may, similarly, be explained, at least in part, by PKC mediated phosphorylation of such components as vinculin (Werth et al. (1983)). This is also true for the changes in glucose transport (Whetton et al. (1986); Witters et al. (1985)).

In addition, PKC activators have been shown to alter the expression of a number of proteins by influencing their level of transcription. It has been shown that the tumour-promoting phorbol ester, phorbol 12-myristate, 13-acetate (PMA), increases the transcription of genes for the IL-2 receptor, T3 and T cell antigen receptor in human T leukemic cell lines (Shackelford et al. (1987)) and induces the expression of the growth cycle-dependent gene, ornithine decarboxylase (O'Brien et al. (1976)). It also increases EGF receptor production in human breast carcinoma cells (Bjorge and Kudlow et al. (1987)) and the production of two secreted glycoproteins, mitogen regulated protein (MRP) and major excreted protein (MEP) in 3T3 fibroblasts, its ability to increase the production of these glycoproteins correlating with

its capacity to stimulate DNA synthesis (Fienup et al. (1986)). PKC activation also stimulates the production of IL-1 in peritoneal macrophages (Katakami et al. (1986)) but decreases the expression of glycophorin (Siebert and Fukuda (1985)) and phosphoenolpyruvate carboxykinase (Chu and Granner (1986)) in human leukemic K562 cells and rat hepatoma H4IIE cells, respectively.

A study in NIH 3T3 cells, treated with PMA, has indicated that PKC activation can alter the expression of two classes of genes, those that do not require prior protein synthesis for transcription and those that do require the production of new proteins (Rabin et al. (1986)). In the latter case PKC is obviously not acting directly on the DNA. In the former case, however, it is not yet clear whether PKC actions are mediated by its ability to phosphorylate other enzymes such as RNA polymerase II (Chuang et al. (1987)) or are due to direct interaction of PKC with enhancer sequences within the genome. Those genes, belonging to the first group, include the cellular proto-oncogenes c-myc and c-fos which have been shown by many groups to be activated by PKC. (Kaibuchi et al. (1986)); Blackshear et al. (1987); Coughlin et al. (1985)). Genes, such as that for MEP, belong to the second class. Interestingly, it has been shown that elevations in $[Ca^{2+}]_i$, produced by $Ins(1,4,5)P_3$, can also induce c-myc transcription, independently of PKC activation (Kaibuchi et al. (1986)) and that prolactin gene transcription is promoted by PKC activators in combination with an elevation in intracellular free Ca^{2+} concentration (Murdoch et al. (1985)).

Other targets of PKC are identified daily, but it is clear from the nature of targets already known that PKC probably occupies a central role in cell proliferation.

1.C.2.4.6 Down-Regulation and Negative Feedback:

In addition to its positive role in signal transduction, PKC is also thought to be involved in the termination of second messenger production through negative feedback inhibition. It has been shown in many cell types, including fibroblasts (L'Allamain et al. (1986)), platelets (Watson and Lapetina (1985)), astrocytomas (Orellana et al. (1985)) and cultured smooth muscle cells (Brock et al. (1985)), that pre-incubation with tumour promoting phorbol esters inhibits agonist-induced stimulation of phosphoinositide hydrolysis and Ca^{2+} mobilization. That this effect is mediated by PKC is supported by the observation that inhibitors of the kinase prevent phorbol ester mediated inhibition (Tohmatsu et al. (1986)). The exact means by which this occurs, however, is not yet fully understood. In some cases, inhibition may be due to a decrease in agonist-receptor binding but, in others, modification of agonist binding is clearly not involved (Brock et al (1985)) and PKC is thought to act by directly influencing receptor-coupled phospholipase C activity. Accordingly, a recent report concerning formyl-methionyl-leucyl-phenylalanine (FMLP) stimulation of PIP_2 hydrolysis in human leukemic cells, has suggested that PKC activation in intact cells impaired the coupling of both the receptor to the GTP-binding protein and of the GTP-binding protein to phospholipase C (Kikuchi et al. (1987)).

Protein Kinase C has also been shown to have a negative effect on the second messengers produced by phosphoinositide hydrolysis. Its ability to phosphorylate the enzyme, inositol trisphosphate 5'-phosphomonoesterase (Connolly et al. (1986)), leads to an increase in phosphatase activity and therefore to the breakdown of Ins(1,4,5)P_3 (and possibly Ins(1,3,4,5)P_4). Furthermore, studies, concerning the association of PKC with the membrane, have indicated that the tight membrane-PKC complex, formed in the presence of phorbol esters, can be dissociated when ATP is added (Wolf et al. (1985a)). This ATP effect cannot be reproduced by other nucleotides, but requires $MgCl_2$ and is accompanied by the phosphorylation of PKC itself, suggesting perhaps, that PKC autophosphorylation may regulate its own association with the membrane. Although it was not conclusively shown that the phosphorylation of PKC (as opposed to some other component) was the phosphorylation event which caused membrane dissociation, it is an interesting proposal, particularly since the different forms of PKC isolated from rat brain have been shown to have different autophosphorylation sites (Huang et al. (1986)).

Evidence in other systems suggests that down-regulation mediated by PKC is not confined to its own receptors, but extends to growth factor receptors whose responses may not be mediated by phosphoinositide hydrolysis. For instance, PKC has been shown to phosphorylate the epidermal growth factor (EGF) receptor with a concomitant decrease in both its tyrosine kinase and growth factor binding activities (Downward et al. (1985); May et al. (1985)). Likewise,

receptors for insulin and insulin-like growth factor are also phosphorylated by PKC, leading in the case of the insulin receptor, at least, to a decrease in the receptor kinase activity (Bollag et al. (1986); Haring et al. (1986)). Somatostatin receptor binding in pancreatic acinar cell membranes (Matozaki et al. (1986)) is also inhibited by the protein kinase C activators, OAG and PMA, which, likewise, act to reduce cell surface transferrin receptor numbers in human leukemic cell lines (Neckers et al. (1986); May et al. (1986)) and leukotriene B_4 receptor binding in neutrophils (O'Flaherty et al. (1986)).

It should also be mentioned that there have been many reports of the so-called down-regulation of tumour-promoting phorbol ester binding sites in response to prolonged treatment of cells with phorbol esters (Fabbro et al. (1986); Gainer and Murray (1985)), suggesting that PKC is itself down-regulated by long term activation. However, this statement should be qualified as there seems to be two distinct phases to the decreases in both amount and activity of PKC.

The initial down-regulation reported in most experiments occurs within 30 minutes of phorbol ester treatment. It may be due, initially, to a desensitization of the enzyme but is thought predominantly to occur by the actual loss of PKC from the membrane as the result of its proteolytic cleavage to the PKM form (Tapley and Murray (1985); Melloni et al. (1985); Chida et al. (1986)).

However, when cells are treated with phorbol esters for an extended period (>12 hr), PKC down-regulation caused by

proteolytic cleavage is followed by a more permanent down-modulation of the enzyme (Fabbro et al. (1986); Gainer and Murray (1985)). A study by Fabbro et al. (1986) indicated that treatment of human breast cancer cells for 72 hours with 100nM PMA, resulted in the complete loss of PKC from the membrane within 2 hours, followed by a slower but total disappearance of the 60kDa and 50kDa (PKM) degradation products within 12 hours. No PKC of any type was detected for some time thereafter, and normal cellular levels of the enzyme were not apparent until at least five days after phorbol ester removal. The fact that the presence of phorbol esters did not lead to the continuous translocation and breakdown of PKC over the 72 hour exposure period, suggests that there may be some negative feedback system operating at the level of the genome whereby the finite cytosolic pool of PKC, once depleted, is no longer replenished.

A recent study which examined this latter type of PKC down-modulation in neuronal cells (Mattingly et al. (1987)) has indicated that the extent of PKC depletion depends, not only on the time of phorbol ester treatment, but also the type (PMA vs PDBu) and concentration of phorbol ester used, suggesting once again that different types of PKC have different susceptibilities to down-modulation mediated by phorbol ester. It is less likely, however, that this second phase of PKC down-modulation has any physiological significance, considering that the PKC-mediated negative feedback of phosphoinositide hydrolysis would prevent the rapidly metabolised DAG from remaining within the membrane for such an extended period. Nevertheless, the depletion of PKC, by

prolonged phorbol ester exposure, is useful experimentally as it has been employed to make "PKC-deficient" cells which can then be used to assess the importance of this enzyme in mediating the positive effects of agonist-receptor activation.

1.C.2.4.7 Role of Protein Kinase C in Mediating Cell Growth:

It is clear that an elevation in cytosolic free Ca^{2+} has a role in mediating the mitogenic signal, and the ability of $Ins(1,4,5)P_3$ to raise $[Ca^{2+}]_i$ in a fashion that mimics the growth factor response, makes it a logical candidate as a second messenger (Berridge (1987)). This natural inference cannot, however, be made for the DAG/PKC arm of the pathway. Although increased protein phosphorylation is also an early event in mitogenesis, the identity of many of the target proteins is unknown. Even if it may be demonstrated that protein kinase C is capable of phosphorylating at least some of these proteins, the overlap of substrate specificity, between kinases, prevents an immediate assessment of the importance of PKC mediated phosphorylation in generating the growth signal.

However, indirect but convincing evidence, that PKC is actively involved in the growth response, comes from many reports correlating the presence of an active, membrane-bound PKC with cell division and the ability of agents which activate PKC to stimulate cell growth. For example, in the study by Fabbro et al. (1986) mentioned earlier, in which human breast cancer cells were treated for extended periods with PMA, it was shown that the time at which no cellular PKC activity was

detected, coincided with the minimum PMA exposure time required for inhibition of growth. Accordingly, the resumption of cell cycling, after PMA removal, was accompanied by the re-appearance of PKC activity. Similarly, calcium deprivation has been shown to inhibit the proliferation of Balb/c 3T3 fibroblasts with an accompanying loss of PKC from the particulate fraction. Re-addition of Ca^{2+} to the medium resulted in a rise in membrane PKC activity which parallelled the increase in DNA synthesis (Donnelly Jr. et al. (1985)).

In addition, investigations into the proliferation of confluent rat liver epithelial cells (Boynton et al. (1985)) indicated that serum-stimulated transition from G_o to G_1 and from G_1 to S required high levels of extracellular calcium and was accompanied by an increase in membrane-associated PKC. Moreover, the requirement for calcium was lost when cells were treated with PMA, suggesting that PKC helps regulate the transition of these cells through the G_1 phase.

Other evidence that PKC translocation to the membrane can accompany mitogenic stimulation comes from studies with B lymphocytes (Guy et al. (1986)) and T lymphocytes (Averdunk and Günther (1986)) and those using purified lymphokines with factor-dependent cell lines. In the latter reports it was not only shown that interleukin-2 (Farrar and Anderson (1985)) and interleukin-3 (Farrar et al. (1985)) could stimulate the rapid but transient association of PKC with the membrane, but also that the amount of membrane-associated C-kinase correlated with the amount of DNA synthesis. Likewise,

studies, using human and mouse (Adamo et al. (1986)) fibroblasts, have indicated that PKC activity and subcellular distribution undergo spontaneous changes in accordance with the proliferative state of the cell.

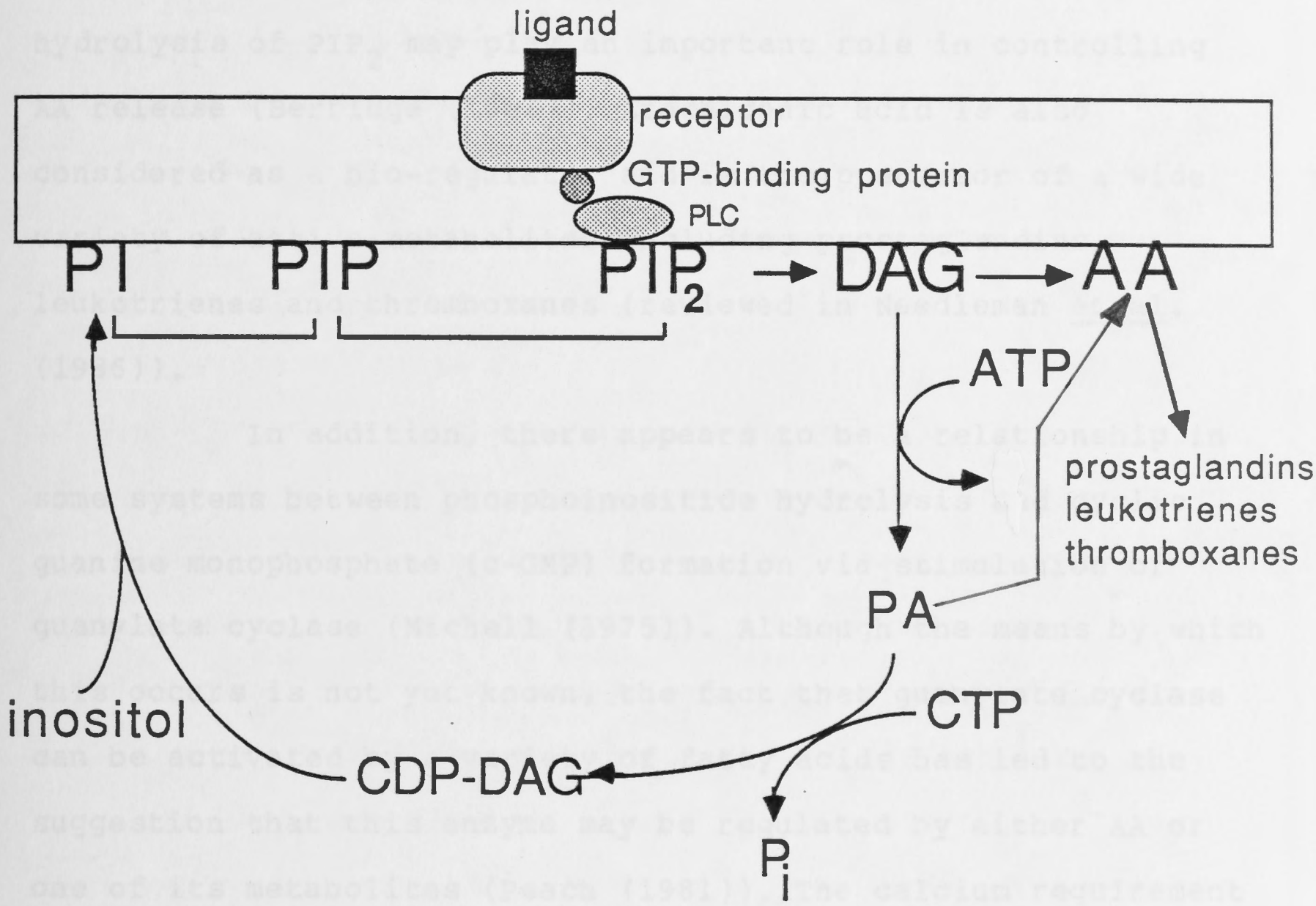
1.C.2.4.8 Metabolism of Diacylglycerol:

Diacylglycerol is normally not found free in the plasma membrane and, in general, appears only transiently, due to such events as receptor driven phosphoinositide hydrolysis (Lapetina et al. (1981)). Once formed, it is rapidly broken down by one of two major pathways. It may be either phosphorylated by 1,2-diacylglycerol kinase to form phosphatidic acid (PA) or cleaved by diacylglycerol lipase, releasing arachidonic acid (AA). (See Diagram 1.6.)

Phosphatidic acid formation seems to be the dominant route under normal conditions (Lapetina et al. (1981)) and PA itself has been proposed as a bio-regulator due to its ability to gate Ca^{2+} across the plasma membrane (Putney et al. (1980); Gerrard et al. (1979); Brass and Laposata (1987)). Like DAG, it can be further metabolised in one of two ways. By combining with cytidine triphosphate (CTP) via CTP-phosphatidate: cytidyl transferase, it is converted to CMP-PA which can accept a free myo-inositol, forming the means by which DAG is re-cycled back to the phosphoinositides (Lapetina et al. (1973)). Alternatively it may be acted upon by a PA-specific phospholipase A_2, releasing arachidonic acid (Billah et al. (1981)).

DAG can also be converted directly to arachidonic acid by a DAG lipase (Bell et al. (1979)). Although the fatty

Figure 1.6 Metabolism of Diacylglycerol



PIP_2 - phoshpatidylinositol (4,5) bisphosphate
PIP - phosphatidylinositol (4) phosphate
PI - phosphatidylinositol
DAG - diacylglycerol
PA - phosphatidic acid

acid composition on the 2-position of the phosphoinositides is usually AA, it can also be linoleate or oleate. However, during agonist stimulation, there appears to be a preferential degradation of molecules such that DAG always carries AA on the 2-position (Bell *et al*. (1979)), prompting the suggestion that hydrolysis of PIP_2 may play an important role in controlling AA release (Berridge (1984)). Arachidonic acid is also considered as a bio-regulator and is the precursor of a wide variety of active metabolites including prostaglandins, leukotrienes and thromboxanes (reviewed in Needleman *et al*. (1986)).

In addition, there appears to be a relationship in some systems between phosphoinositide hydrolysis and cyclic guanine monophosphate (c-GMP) formation via stimulation of guanylate cyclase (Michell (1975)). Although the means by which this occurs is not yet known, the fact that guanylate cyclase can be activated by a variety of fatty acids has led to the suggestion that this enzyme may be regulated by either AA or one of its metabolites (Peach (1981)). The calcium requirement for c-GMP formation could be met by changes in intracellular Ca^{2+} homeostasis modulated by the $Ins(1,4,5)P_3$ arm of the pathway.

1.C.2.5 Synergism Between the Two Arms of the Phosphoinositide Pathway and their Interaction with other Growth Signals in the Mitogenic Response

One of the most intriguing aspects of the phosphoinositide signal transduction system is that both molecules generated by the hydrolysis of PIP_2 can be assigned a second messenger function (Berridge (1987)). The aqueous soluble $Ins(1,4,5)P_3$ is proposed to act by increasing $[Ca^{2+}]_i$ and DAG is thought to function by activating the enzyme, protein kinase C. (Berridge (1984)). Although there is evidence to suggest that both an increase in $[Ca^{2+}]_i$ (Poggioli et al. (1986)) and the activation of PKC (Farese et al. (1985)) can be generated independently within cells, via mechanisms that do not involve phosphoinositide hydrolysis, it is also clear that when these signals are generated in combination they act synergisticly to mediate a biological response (Berridge (1987)).

The interaction between the two arms of the pathway is demonstrated by the documented effects of $[Ca^{2+}]_i$ on the activity and subcellular distribution of PKC (Bell (1986)) and by the ability of PKC activation to not only modulate the generation (Bianca et al. (1986)) and catabolism (Connelly et al. (1986)) of $Ins(1,4,5)P_3$ but also to act upon calcium channels to directly modulate $[Ca^{2+}]_i$ (Strong et al. (1987); Lagast et al. (1984)). Many examples of how these two systems act in concert to generate a biological response have

been reported (Kikkawa and Nishizuka (1986); Berridge (1987)) but perhaps the clearest indication of the synergistic role of these two signals in stimulating mitogenesis is given by the finding that lymphocytes can be stimulated to initiate DNA synthesis by pharmacological agents which mimic the signals generated by phosphoinositide hydrolysis. Treatment of T lymphocytcs with a combination of calcium ionophore (to raise $[Ca^{2+}])_i$ and phorbol 12-myristate 13-acetate (PMA) (to activate PKC) leads to DNA synthesis whereas neither agent alone (administered in concentrations consistent with their ascribed action) could generate a fully mitogenic response (Truneh et al. (1985)). However, it has also been shown that when T lymphocytes are depleted of macrophages they will not re-initiate DNA synthesis in the presence of calcium ionophore and PMA without the additional presence of the co-mitogen, phytohaemagglutinin. (Kaibuchi et al. (1985)). This implies that although the phosphoinositide pathway may be actively involved in generating a mitogenic signal, it is not sufficient, by itself, to initiate DNA synthesis, a conclusion which is logical if one is to propose a multistep system in the regulation of cell growth (see next section).

It has been found that when most known mitogenic agents are added singly to quiescent cells in serum-free medium they fail to stimulate cell division. However, when such agents are added in specific combinations they show striking synergism (Rosengurt and Mendoza (1985)). Therefore, by the co-ordinate examination of the molecular mechanisms by which these agents act, attempts are being made to determine the set of signals

necessary for cell division. (Rosengurt and Mendoza (1985); Metcalfe et al. (1985)).

A study in fibroblasts (Rosengurt and Mendoza (1985)) has indicated that the mitogenic agents for these cells can be divided into at least two groups based on the pattern of early signals they generate. The first group, exemplified by vasopressin, is catagorized by the ability to generate monovalent anion fluxes and to activate PKC. The second group containing prostaglandin E_2, failed to initiate any of the ion fluxes associated with mitogenesis (see Section 1.B) but resulted in a prolonged increase in c-AMP. Combination of factors within a group did not result in DNA synthesis but combinations between groups can lead to a full mitogenic response. Moreover, the only agents which were found to stimulate DNA synthesis, when added alone, were platelet-derived growth factor (PDGF) and bombesin, both of which activate phosphoinositide hydrolysis and raise c-AMP levels.

In addition, PDGF has been shown to stimulate the activation of the tyrosine protein kinase activity associated with its receptor (Ek and Heldin (1984)). Phosphorylation of proteins on tyrosine residues is also one of the early events associated with mitogenesis and, because a number of growth factor receptors, including those for PDGF, epidermal growth factor (EGF), and insulin have been shown to possess tyrosine kinase activity, it has been proposed that this may be an additional pathway for the transfer of mitogenic signals into the cell (reviewed in Hunter and Cooper (1985)). None of these

three signals (phosphoinositide hydrolysis, elevation of c-AMP and activation of tyrosine kinase activity) are, however, essential for growth, as a maximal mitogenic response can be generated with any one of these signals missing.

It is clear that these separate signals do not act independently but interact with one another to produce an overall mitogenic response. In some cases this leads to the potentiation of one of the signals. For example, although insulin does not generate PIP_2 hydrolysis on its own, it acts synergisticly with bombesin to enhance DNA synthesis and inositol phosphate formation (Heslop et al. (1986)). In addition, activation of PKC has been shown to increase the production of c-AMP via modulation of adenylate cyclase (Summers et al. (1988)) and to phosphorylate, and apparently suppress, the function of the inhibitory GTP-binding protein N_i coupled to adenylate cyclase (Katada et al. (1985)). However, in other cases it appears that the signals are antagonistic. Protein Kinase C activation has been shown to decrease the affinity of EGF (Downard et al. (1985)) and insulin (Bollag et al. (1986)) receptors for their agonists with a concomitant decrease in tyrosine kinase activity. Moreover, the c-AMP and phosphoinositide pathways are counteractive in some cells (see Kikkawa and Nishizuka (1986); Kaibuchi et al. (1984); Gainer and Murray (1986)). Even more confusing are the reports that both c-AMP generation (Katsaros et al. (1987)) and PKC activation (Issandou and Darbon (1988)) have been associated with the inhibition of cell proliferation in some systems.

Perhaps some of these apparent contradictions are due to differences in the duration of the stimulus required to produce the different responses. A key feature in the action of most mitogenic agents is that they have to occupy their receptors for hours before they stimulate increased DNA synthesis in the cell population (Rosengurt and Mendoza (1985)). A large transient increase in the cellular levels of c-AMP which is traditionally considered as the means by which this molecule acts in signal transduction (Robison et al. (1971)), is not sufficient to act as a co-mitogenic stimulus in fibroblasts. Instead, c-AMP concentration must remain elevated for hours (Rozengurt (1982)). Likewise, a transient exposure of cells to vasopressin or phorbol esters is not sufficient to stimulate mitogenesis (Dicker and Rozengurt (1981)). Moreover, although short term activation of PKC leads to the down-modulation of EGF receptors, when quiescent cells are exposed for a number of hours to PMA or a synthetic DAG, these PKC activators act synergisticly with submitogenic levels of EGF to stimulate DNA synthesis (Davis et al. (1985)). This is not to suggest, however, that phosphoinositide hydrolysis is continuous in the presence of the appropriate agonists since it has been clearly documented that this signal transduction system is under negative feedback control (see Section 1.C.2.4.5). Nevertheless, it is possible that phosphoinositide breakdown may oscillate in the presence of agonists. (Cuthbertson and Cobbold (1985); Berridge (1987)) and that sustained oscillations are required for the generation of the mitogenic response.

It is clear that stimulated phosphoinositide hydrolysis is associated with the actions of a number of mitogens including PDGF, bombesin, vasopressin phytohaemagglutinin and thrombin. (Berridge (1987a)). In addition, the second messengers, produced by this hydrolysis, are capable of mediating many of the early events which are responsible for ultimate cell division including increased $[Ca^{2+}]_i$, (Berridge (1984)), increased monovalent ion fluxes (Vara et al. (1985)) and increased protein phosphorylation (Kikkawa and Nishizuka (1986)). Moreover, PKC and $[Ca^{2+}]_i$ have both been shown to stimulate the transcription of several genes, the expression of which correlates with the progression through the cell cycle (Rabin et al. (1986)). All this information suggests that the phosphoinositide pathway is one of the means by which the positive growth signals, delivered to the cell by growth factors, are converted to a mitogenic response.

1.D CELLULAR GROWTH REGULATION

The phosphoinositide pathway forms one of the means by which the information, delivered to the cell by growth factors, is converted into a positive growth signal inside the cell (reviewed in Berridge (1987a)). It has also been shown that the messages delivered by this pathway must be complemented by an additional positive signal before a cell is committed to divide (Kaibuchi et al. (1985); Rozengurt and Mendoza (1985)), indicating that cell proliferation is a multifaceted process which may be regulated by co-ordinate

presentation of the appropriate signals. However, it is now apparent that such regulation is not simply dependent on the presence or absence of growth inducers, but is a highly complex and dynamic process involving both positive and negative factors acting in concert to regulate cell growth. This concept, termed the Yin and Yang of growth control (Sager quoted in Marx (1986); Pardee (1987)), illustrates the balanced nature of normal cell growth and allows a conceptual understanding of how an imbalance of such factors can lead to alterations in growth regulation.

Cells which have left the growth cycle and have become quiescent are in, what is termed, the G_o state (see Section 1.A "Cell Cycle" - diagram 1.1). Factors, which determine whether the cell will remain in this state or undergo cell division, act at two main events, both of which have been proposed to be controlled by yes/no "switches" (Pardee (1987)). The first involves the re-entry of the cell into the cell cycle and occurs at the transition from the G_o state to the G_1 phase of the cell cycle in a process known as competence (Pardee (1987)). A cell is made competent to divide by such factors as platelet derived growth factor (PDGF) (Stiles et al. (1979)). Approximately 12 hours later a second choice is made in the competent cells. Under the influence of other factors, such as epidermal growth factor (EGF) or insulin-like growth factor-1 (IGF-1), cells will either progress towards DNA synthesis or, alternatively, go back into quiescence. This second switching event is made at, what is termed, the restriction point about two hours before the onset of DNA

synthesis (Pardee et al. (1986)). After this, cells are no longer under the controlling influences of growth factors and it is presumed that once cells have replicated their DNA, they are committed to division since most cells in the animal are diploid when in G_o (Pardee (1987)).

The activation of these "switches" is dependent on the outcome of the interplay between the opposing positive and negative forces which regulate the cell cycle. Conceptually, if a cell or its environment is manipulated so that the balance of such forces is permanently tipped to one side, then the switch becomes "stuck" in the one position and an element of growth control is lost. When both switches are "jammed" in the "on" position, the cell can readily transit the cell cycle without restriction. It is not clear, however, whether this change is sufficient to generate a tumour in vivo since there is growing evidence for the existence of additional negative growth signals, intrinsic to the cell genome, which may operate at positions other than the growth cycle switches to prevent unrestricted cell division and ensure the programmed differentiation of the cell (Stanbridge (1985); Harris (1985)).

Accordingly, both in vivo and in vitro evidence suggests that tumourigenesis is a multistage process (Foulds (1954); Land et al. (1983a)), the endpoint of which, for the purposes of this discussion, is the production of a cell which is capable of forming a tumour in a suitable animal host. The additional ability of such cells to spread throughout the body via a process known as metastasis is also a multistep process (reviewed in Poste and Fidler (1980); Ling et al. (1984)) not considered further.

Cancers of every tissue type have now been found in man and many of these, as well as those developed experimentally in animal models, have been extensively studied both *in vivo* and *in vitro* in order to determine the molecular basis of growth regulation. Some of the factors, both positive and negative, which have been shown to influence cell growth will now be discussed with reference to their action in the production of tumourigenic cells.

1.D.1 POSITIVE FACTORS REGULATING CELL GROWTH:

Cell proliferation is usually initiated by the binding of peptide growth factors to their cell surface receptors, resulting in the activation of intracellular second messenger systems (Rozengurt and Collins (1983)). Under normal circumstances, the magnitude and duration of the positive signal produced, is regulated by controlling either the number of agonist-receptor complexes formed or the ability of such complexes to generate second messengers. It is not surprising, therefore, that events which lead to a long term increase in either:-

(1) availability of growth factors,

(2) the number of activated receptors, or

(3) the generation of second messenger molecules,

have all been shown to be capable of disrupting cellular growth control with a resultant change in growth state of the cell.

Many of the so-called cancer genes or oncogenes have been shown to act in this fashion (Marshall (1986)). Oncogenes are the transforming genes carried by retroviruses (Duesberg

(1987)). Such viruses can produce tissue specific tumours upon infection of animal hosts and, therefore, the genetic material they carry has been widely studied in the hope of discovering the genetic basis of cancer (reviewed in Weinberg (1985); Bishop (1986); Marshall (1986)). More than 30 oncogenes have now been found and their protein products characterized into groups depending on their subcellular location, biochemical activity and proposed cellular functions (Marshall (1986); Weinberg (1985)). Examination of DNA sequence of the oncogenes indicates that many are hybrid genes that consist of truncated cellular genes (known as proto-oncogenes) recombined with regulatory, and often coding, elements from truncated retroviral genes.

Although the function of all the cellular proto-oncogenes is not yet known, many have, been shown to code for proteins actively involved in the mitogenic response (Marshall (1986)). It should be noted, however, that the oncogenes are distinct from their cellular counterparts and the proposed oncogene concept, which states that proto-oncogenes are latent cancer genes which can be activated from within the cell to form virus-negative tumours (Weiss (1986)), is currently losing favour (Duesberg (1987); Klein and Klein (1986)). Moreover, although retroviral innoculation can lead to tumourigenesis in vivo, laboratory studies have indicated that transfection of normal primary cell cultures with a single activated oncogene, does not lead to the production of tumourigenic cells. (Land et al. (1983); Newbold and Overwell (1983)) These cells show altered growth characteristics but

transfection with at least one additional, complementary oncogene is necessary before such cells produce tumours. This is consistent with the proposal that tumourigenesis is a multi-step process and indicates, that since particular combinations of oncogenes are required for the production of a tumour, the nature, as well as the magnitude of the changes in cell function, are important. Interestingly, complementary oncogenes belong to different catagories acting either at the plasma membrane or within the nucleus (Weinberg (1985)), and may be associated with the key events occurring after the switch points during G_1.

1.D.1.1 Increased Availability of Growth Factors and the Altered Growth State

An example of oncogene activation, resulting in the over-production of growth factors, comes from work studying the transfection of cells with Simian Sarcoma Virus (SSV) which carries the oncogene v-sis. The v-sis gene encodes a protein which is very similar or identical to that of platelet derived growth factor (PDGF). (Waterfield et al. (1983); Doolittle et al. (1983)) Since SSV transfected fibroblasts are capable of responding to PDGF, the transfected cells can undergo what is termed "autocrine" stimulation of cell proliferation, becoming independent of exogenously supplied factor. However, although transfection of continuous cell lines such as NIH 3T3 cells, with either SSV or a special construct of cellular sis (c-sis) coupled to appropriate transcriptional and translational control elements, can lead to the production of tumourigenic

lines (Clarke et al. (1984)), the transfection of primary fibroblasts with SSV results in a cell that displays a transformed morphology but will not form tumours.

Further examination of autocrine stimulation phenomenon has indicated that inappropriate expression of the basic fibroblast growth factor gene can also lead to autonomous cell proliferation with the permanent acquisition of the transformed phenotype (Neufeld et al. (1988)). In addition, a study by Lang et al. (1985) in which the gene for the haemopoietic growth factor, granulocyte-macrophage-colony stimulating factor (GM-CSF), was transfected into a factor-dependent murine cell line (FDC-P1) indicated that such cells synthesised and secreted GM-CSF, grew independently of exogenous GM-CSF and, unlike the parental line, formed tumours in the syngeneic mice. However, as with the case of SSV, transfection of normal haemopoietic cells with the gene construct, failed to make the cell tumourigenic. This suggests that autocrine stimulation of proliferation is not sufficient to make normal cells tumourigenic but that it does tip the balance of an already partially deregulated cell.

1.D.1.2 Increased Number of Functional Receptors and the Altered Growth State

A second way in which factors, which disrupt cell growth, may act, is by changing the number and function of cell surface growth factor receptors. Many of the known growth factor receptors, including those for PDGF, EGF, insulin-like growth factor (ILF-1) and monocyte growth factor (CSF-1), have

tyrosine kinase activity associated with their cytoplasmic face, the activity of which is regulated by agonist binding (Ramachandran and Ullrich (1987)). Such kinases are thought to generate second messages in an alternative or complementary manner to the phosphoinositide pathway. No less than 7 of the known oncogenes have such tyrosine kinase activity and it has been proposed that they may represent captured receptor genes. (Hunter and Cooper (1985)). Support for this comes from the finding that v-erb B, the oncogene carried by the avian erythroblastosis virus (AEV), has a sequence corresponding to a truncated EGF receptor (EGF-R) (Ullrich et al. (1984)). It contains stretches of the transmembrane and tyrosine kinase sequence of EGF-R but lacks the amino-terminal EGF binding domain and the extreme carboxyl terminal cytoplasmic domain. These deletions result in a viral oncogene product that expresses tyrosine protein kinase activity constitutively, independent of external regulation by EGF. In addition, it has been shown that c-fms, the cellular counterpart of v-fms, carried by the feline sarcoma virus (FSV), is the receptor for CSF-1 (Sherr et al. (1985)) and that the amino acid sequence for the insulin receptor has limited sequence homology to v-ros (cited in Ramachandran and Ullrich (1987)).

In addition, an in vivo demonstration of the influence of receptor number on growth control is given in a study by Gill et al (1987), examining the relative growth rate of clonal human epidermoid carcinoma cells, A431, containing different amounts of EGF receptor gene amplification and protein expression. A direct correlation between the rate of

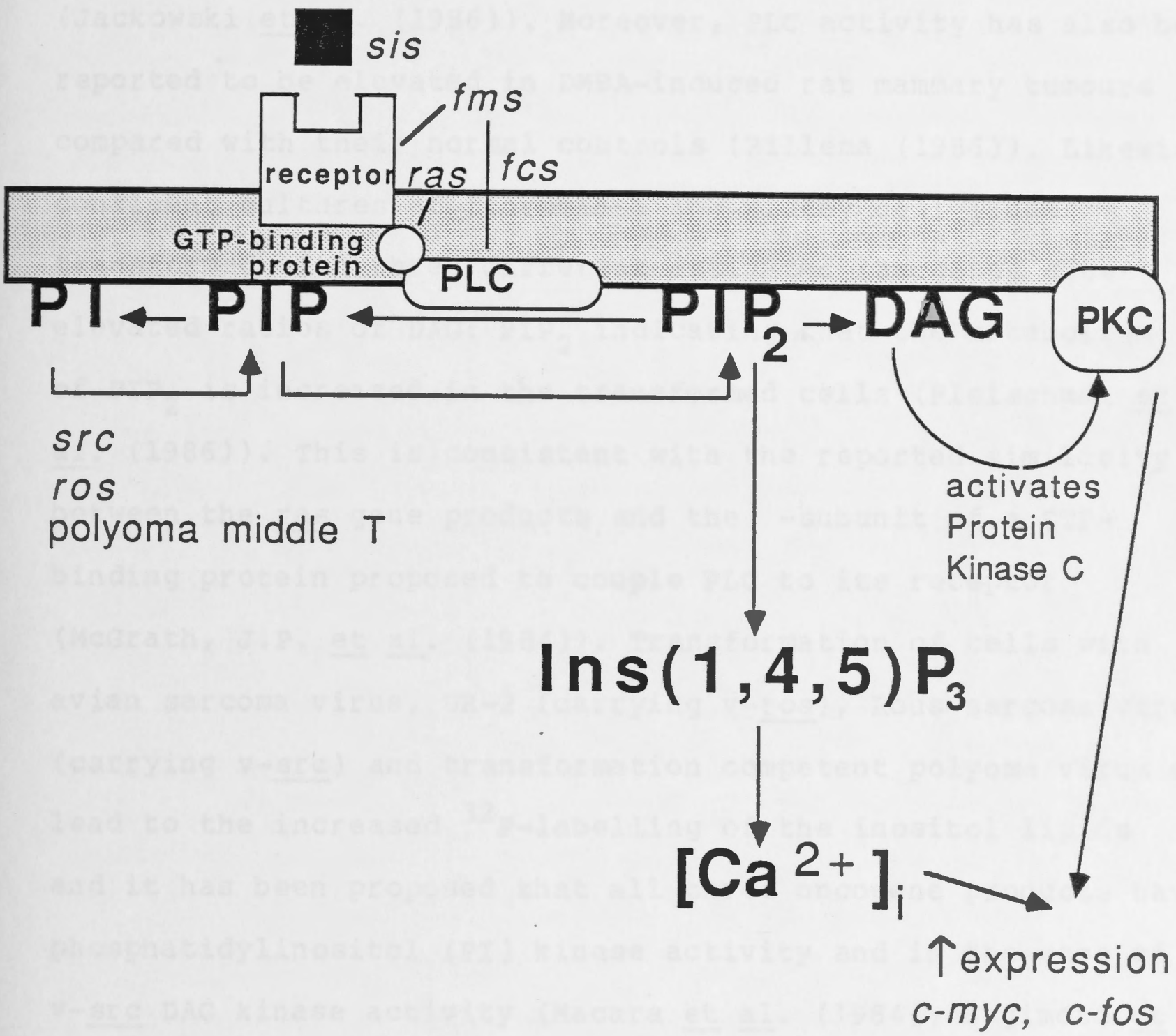
tumour growth and EGF-R concentration was found, supporting previous cell culture studies which quantitated the relationship between activated EGF receptors and cell proliferation.

1.D.1.3 De-regulation of Second Messengers and the Altered Growth State

A third stage at which the positive signals induced by growth factors can be manipulated, is at the level of the intracellular biochemical pathways activated by growth factor receptor binding. In the case of the tyrosine kinase mediated signal, the second messenger system is so tightly coupled to the receptor that both are on the same molecule (Ushiro and Cohen (1980)) and therefore a deregulation of the receptor also leads to the deregulation of the second messenger system. In the case of the phosphoinositide pathway, however, although phospholipase C is coupled to growth factor receptors via a GTP binding protein (Cockcroft (1986); Litosch and Fain (1986)), the association is not as tight, and the efficiency of the system can be modified without a change in the receptor molecule. By manipulating the components of the signal transduction system independently of the receptor, the actions of more than one growth factor can be affected. It is not surprising, therefore, that a number of steps in the phosphoinositide pathway are targeted by oncogenes (Michell (1984) (see diagram 1.7)) and have been shown to be deregulated in several chemically induced tumours (Rillema (1986); Kuboto et al. (1986)).

Figure 1.7

Oncogenes and the Phosphoinositide Pathway



It has been shown that transformation of a mink lung epithelial cell line with feline sarcoma viruses, carrying either v-fms or v-fes, results in significantly higher levels of guanine-nucleotide activated-PIP_2-specific-phospholipase C (PLC) activity in the transformed cells leading to elevated steady state levels of inositol phosphates in these cells (Jackowski et al. (1986)). Moreover, PLC activity has also been reported to be elevated in DMBA-induced rat mammary tumours compared with their normal controls (Rillema (1986)). Likewise, confluent cultures of fibroblast and kidney cell lines transformed with three different activated ras genes show elevated ratios of DAG: PIP_2 indicating that the metabolism of PIP_2 is increased in the transformed cells (Fleischman et al. (1986)). This is consistent with the reported similarity between the ras gene products and the -subunit of a GTP-binding protein proposed to couple PLC to its receptor (McGrath, J.P. et al. (1984)). Transformation of cells with avian sarcoma virus, UR-2 (carrying v-ros), Rous sarcoma virus (carrying v-src) and transformation competent polyoma virus all lead to the increased ^{32}P-labelling of the inositol lipids and it has been proposed that all three oncogene products have phosphatidylinositol (PI) kinase activity and in the case of v-src DAG kinase activity (Macara et al. (1984); Sugimoto et al. (1984); Kaplan et al. (1986)). This has, however, been disputed (Sugano and Hanafusa (1985); MacDonald et al. (1985)) and these oncogenes may act, instead, to influence the cellular kinases. SV40 transformed fibroblasts have elevated unstimulated levels of inositol phosphates (Villereal et al.

(1985)) and kidney tumour cells, displaying an abnormally increased expression of the proto-oncogene c-myc, showed a very marked enhancement of ^{32}P labelling in CDP-diacylglycerol. (Kubota et al. (1987). Moreover, the tumour promoting phorbol esters have been shown to activate the enzyme protein kinase C, both in vivo and in vitro as have a number of other tumour promoters such as teleocytin and Aplasia toxin (Nishizuka (1984)).

In addition, it has been shown that second messengers generated by different pathways are capable of activating a common set of cellular genes whose expression appears necessary for the transit of the two switch points in the cell cycle mentioned earlier (Pardee (1987)). Several of these mitogenically regulated genes including c-myc, c-fos and c-ras have oncogene counterparts.

1.D.2 NEGATIVE FACTORS REGULATING CELL GROWTH:

As illustrated above, a sustained increase in the intensity of the positive signals which promote cell division, can lead to an alteration in growth state of a cell and thereby contribute to its progression to tumourigenicity. However, there is also evidence that a decrease in the level of negative growth signals can have the same effect (Marx (1986)). Conceptually, there are two ways in which such negative signals can act, either by inhibiting the actions of the positive signals or by actively directing the cell away from proliferation into a non-proliferative state (differentiation?).

Evidence that the first mechanism is operating is

demonstrated by such phenomena as receptor down-regulation and inactivation of key enzymes involved in signal transduction. These systems are often inbuilt into the normal cell division process as part of a negative feedback loop. For example, protein kinase C inhibits phospholipase C (Kikuchi et at. (1987)).

Additional support for the existence of negative growth signals is given by the relatively recent discovery and characterization of a number of growth inhibitory substances, including transforming growth factor beta (TGFβ) (Roberts et al. (1985)), beta-type interferon (IFβ) (Mehmet et al. (1987)) and tumour necrosis factor (TNF), which have been shown to have antiproliferative effects in some cell types. Although these factors act from outside the cell, they, too, may be part of a negative feedback loop, since for example, PDGF has been shown to stimulate interferon production in fibroblasts (cited in Marx (1986)). The importance of such factors in regulating normal cell growth is illustrated in a study by Moses et al. (1985) examining the actions of TGFβ on carcinoma cells. It has been shown that many carcinoma cell lines grew well in serum (containing inactive TGFβ) but are inhibited by active TGFβ, implying that the carcinoma cells have lost the capacity to activate TGFβ. Since most cells, including carcinoma cells, produce TGFβ in an inactive form, this inability to generate a negative signal may have contributed to the tumourigenic phenotype. The way these agents work at the molecular level is not yet known. In general, they are not considered to act by inducing terminal differentiation although both TGFβ and TNF

have been shown to promote differentiation in some cell types (Markovac and Goldstein (1988); Takeda et al. (1986)).

Probably, the most conclusive support for the idea that negative signals operate to prevent uncontrolled proliferation in vivo, comes from the study of somatic cell hybrids derived from the fusion of normal diploid cells and tumourigenic cell lines (Stanbridge et al. (1982); Klinger (1980); Harris (1985)). It has been shown in human and rodent systems that when malignant cells, defined by their ability to form tumours in a compatible host, are fused with diploid cells of the same (Harris et al. (1969); Stanbridge (1976)) or different (Stoler and Bouck (1985)) species, the resultant hybrids are non-tumourigenic as long as they retain certain specific chromosomes from the diploid parent. When such chromosomes are lost from the hybrid, the segregant cells are again able to grow in vivo. Although this is seen in inter- as well as intra-specific hybrids, it is more clearly displayed in intra-specific hybrids, particularly of human origin, in which a higher degree of genetic stability is observed (Stanbridge et al. (1982)). The fact that the non-tumourigenic hybrids maintain their tumourigenic potential, which can be re-expressed by loss of specific chromosomes, indicates that the tumourigenic phenotype is being suppressed by some component, presumably carried, in an intact form, by the normal cell genome (Harris (1985)). In addition, it should be noted that the non-tumourigenic hybrids, like their tumourigenic segregants, usually behave as transformed cells in culture displaying abnormal growth characteristics such as lack of

contact inhibition, reduced growth factor requirements and anchorage independent growth (Stanbridge et al. (1982). This indicates that the transformed and tumourigenic phenotypes are under separate genetic control (Stanbridge et al. (1982)).

The observation that re-expression of tumourigenicity is correlated with the loss of specific chromosomes, indicates that specific genetic elements (referred to as tumour suppressor genes (Stanbridge (1985) or antioncogenes (Knudson (1985)) are responsible for suppression of tumourigenicity. The identity of the individual chromosomes has been determined for a number of intra-(Stanbridge et al. (1981)) and inter-specific (Stoler and Bouck (1985)) hybrids and the gene(s) responsible for tumour suppression are currently being sought in order to determine how such elements operate at the molecular level.

Investigations in a murine intra-specific hybrid system indicated that suppressor gene action may be overridden by the oncogene Ha-ras in that NIH3T3 fibroblasts transformed by Ha-ras, are no longer able to suppress tumour formation after fusion with the mouse melanoma derivative, PG19 (Harris (1985)). However, other groups, using different hybrid systems, have shown that suppression of tumourigenicity was observed even when the non-tumourigenic hybrids were expressing the ras gene protein (Benedict et al. (1984)), suggesting that, at least in some systems, repressor genes can override the actions of oncogenes.

Alternatively, it has been proposed that suppressor genes may act by actively promoting the maintenance of a

differentiated phenotype rather than by directly inhibiting proliferation (Harris (1985); Stanbridge et al. (1983)). Support for this comes from histological examination of the N1H3T3 x PG19 hybrids innoculated into compatible hosts (Harris et al. (1985)). It was shown that the non-tumourigenic cells produced a collagenous extracellular matrix in vivo but that the original tumour cells and the tumourigenic segregants derived from the hybrids, grew and produced tumours devoid of an extracellular matrix. Harris (1985) proposed that cell multiplication in vivo ceased when the ability to produce an extracellular matrix was restored. Since the deposition of this matrix precedes the suppression of cell multiplication in the non-tumourigenic innoculates and the establishment of such a matrix is a manifestation of terminal differentiation in fibroblasts, it is reasonable to conclude that the cells stopped dividing in vivo because they underwent terminal differentiation (Harris (1985)). Furthermore, it has been shown by Peehl and Stanbridge (1982) that in HeLa x keratinocyte hybrids, cell multiplication in vivo is suppressed, or at least greatly reduced, in those hybrids in which squamous differentiation and keratinization take place. Interestingly, in the cases documented, the non-tumourigenic cells which, undergo terminal differentiation in the animal host, display the phenotype of the normal parent cell irrespective of the origin of the malignant parental cell (Stanbridge (1985)).

In addition, since differentiation is the process whereby a cell is committed to differentially express a subset of genes in its genome (resulting in a unique functional and

morphological phenotype) it is reasonable to assume that the intracellular factor(s) which direct the selection of such genes and/or the maintenance of that state, would be different for different cell types. Therefore, if suppressor genes are actively involved in the differentiation process, it would be predicted that tumours arising from different cell types would respond to distinct suppressor genes.

In agreement with this, it has been shown that fusions between tumours of different tissue origin showed that carcinoma x carcinoma hybrids maintained their tumourigenicity, but that carcinoma x sarcoma and carcinoma x melanoma crosses produced hybrids which were non-tumourigenic, indicating gene complementation between some tumour types (Stanbridge et al. (1982)). Moveover, a number of human tumours excised from patients have been shown by cytogenetic analysis to have specific chromosomal re-arrangement or deletions characteristic of the malignancy (Knudson (1985)). For example, retinoblastoma, renal cell carcinoma and Wilms' tumour are associated with the loss of both allelles from chromosomes 13 (Murphree and Benedict (1984)),3 (Zbar et al. (1987)) and 11 (Koufos et al. (1984)) respectively.

However, it should also be noted that a liver cancer (hepatoblastoma) and a muscle cell cancer (rhabdomyosarcoma) are also associated with deletion of the alleles on chromosome 11 characterized by Wilms' tumour, indicating that at the tissue level, suppressor genes are not unique. Nevertheless, it has been stated by Cavenee (cited in Marx (1986)) that all three tumours have the appearance of normal embryonic tissue,

maintaining the concept that the tumours are failing to differentiate *in vivo*. The involvement of a locus on chromosome 11 is particularly interesting since it has been shown in the HeLa x fibroblast hybrids, that re-expression of tumourigenicity correlates with loss of chromosome 11 (Srivatsan *et al*. (1986); Kaelbling and Klinger (1986)). Moreover, reintroduction of chromosome 11 is sufficient for the suppression of tumourigenicity in the hybrids (Saxon *et al*. (1986)). Such corresponding findings give support to the contention that the actions of the tumour suppressor genes, observed to operate in the somatic cell hybrid systems, are also operating in diploid cells and that their loss from such cells actively contributes to the formation of a tumour in the animal.

The control of normal cell proliferation, therefore, relies on the maintenance of a balance between positive and negative forces. It has been proposed that increases, in the frequency or intensity of positive signals, act to deregulate cell growth by manipulating one or both of the "switches" associated with the transition from G_o to S phase. However, results from somatic cell hybrid studies indicate that the maintenance of controlled proliferation *in vivo*, also requires the functioning of a third switch, perhaps located outside the cell cycle, which is associated with the progression to the differentiated phenotype. It has been shown that the transfection of normal cells with complementary oncogenes results in the production of tumourigenic lines (Land *et al*.

(1983)). If it is assumed that oncogenes act solely as positive signals modifying events at the $G_o \longrightarrow G_1$ and $G_1 \longrightarrow S$ switches, then it would have to also be assumed that an alternation in the ability of the cell to transit switch 3 and undergo differentiation is not necessary for the production of a tumour. However, it is possible and highly conceivable that, in addition to their ability to promote proliferation, at least some oncogenes act as anti-differentiation factors. For example, it has been shown that transfection of fibroblasts (Arbogast et al. (1977)) or epithelial cells (Falcone et al. (1985)) with the several different oncogenes prevents the expression of a differentiated phenotype. Moreover, the tumourigenic contribution of activated oncogenes can be considered to be differentiation-dependent (Klein and Klein (1986)) in that the transforming ability of an oncogene is dependent on the cell type and the stage of that cell in neoplastic progression. Whether these oncogenes are acting by actively blocking differentiation events or promoting the loss of genes necessary for differentiation remains to be determined.

1.E SCOPE OF THESIS

This thesis examined specific aspects of the phosphoinositide pathway in transformed, non-tumourigenic and transformed tumourigenic human somatic cell hybrids in order to assess the involvement of cell surface signal transduction events in maintaining the tumourigenic phenotype.

Tumourigenicity, as such, can only be defined in an in vivo context. There is no unequivocal way of knowing whether

cells in culture are tumourigenic without introducing them into an animal. Conversely, whilst the cells remain within the animal, it is almost impossible to assess the intracellular biochemical changes that have taken place to make that cell tumourigenic. The advantage of the HeLa X fibroblast human somatic cell hybrid system, first developed by Stanbridge (1976), is that both non-tumourigenic and tumourigenic cell hybrids can be obtained from the one fused cell. Moreover, such cell lines, unlike many hybrid systems, are genetically and therefore, phenotypically stable for an extended period in culture (Stanbridge et al. (1982)). This allows the "in vitro" study of highly related cell lines which are known to differ in their growth behaviour in vivo. In addition, because both tumourigenic and non-tumourigenic cell hybrids display a transformed phenotype in culture, any biochemical differences noted between the two cell types are presumably associated with a defined stage of growth control involving the transition from a disrupted (transformed) to fully deregulated (tumourigenic) growth state.

As seen from the previous section concerning the negative control of growth, the transition from the transformed to the tumourigenic state in this hybrid system correlates with the loss of tumour suppressor genes (Stanbridge 1985). These genes are conceptualized as transacting regulatory elements affecting the expression of unrelated genes (Stanbridge 1985) which are proposed to function by promoting cellular differentiation and growth arrest (Harris (1985); Stanbridge et al. (1983)). However, it has not yet been determined at the

molecular level, how these genes act or what effect their loss has on the expression of positive and negative growth signals within the cell. They may act solely to switch on differentiation genes, activation of which is sufficient in itself to inhibit proliferation. Alternatively, they may have a dual role, not only promoting differentiation but also impinging on the regulation of the growth cycle to down modulate the positive growth signals which occur during normal cell proliferation and which are enhanced or deregulated by activated oncogenes (Michell (1984)). This latter possibility is supported by the observation that the loss of suppressor genes in the human somatic cell hybrids is associated with the expression of a membrane-associated phosphoglycoprotein with protein kinase activity (Der and Stanbridge (1981)).

A number of morphological and biochemical properties of these tumourigenic and non-tumourigenic hybrids have been examined in culture, with particular reference to the cytoskeleton and differentiation markers (Stanbridge et al. (1982), Gowing et al. (1984); and Tellam and Banyard (1986)). One of the most interesting findings in the context of positive growth signals concerns the report by Banyard and Tellam (1985) indicating that the tumourigenic somatic cell hybrids had significantly higher levels of intracellular free calcium $[Ca^{2+}]_i$ than their non-tumourigenic partners. Although these differences are not large and the measurements represent an average for a cell population, evidence from many sources suggests that an elevation in $[Ca^{2+}]_i$ plays an important part in the triggering of mitogen-induced proliferation (Veigl

et al. (1984)) and that changes in $[Ca^{2+}]_i$ occur at various stages throughout the growth cycle (Poenie et al. (1985)). Therefore, the elevated $[Ca^{2+}]_i$ in the tumourigenic hybrids may relate to a change in the growth responsiveness of the cell. There are, of course, many possible causes for this elevation, particularly since the regulation of intracellular calcium levels involves such a complex interplay of events (reviewed in Carafoli (1987)). However, in normal cells, it is now considered that one of the main ways in which mitogens generate the rise in $[Ca^{2+}]_i$ is by activation of receptor driven phosphoinositide hydrolysis (Berridge et al. (1984); Berridge (1987a)).

The phosphoinositide pathway was first proposed by Michell (1975) as the means by which calcium-mobilizing hormones acted and has since been shown to be the signal transduction pathway used by numerous hormones, neurotransmitters and growth factors (Berridge 1986)). Much information has been gained over the last ten years concerning the operation of this pathway and phosphoinositide metabolism has evolved into an exciting and rapidly growing area of research. Its role in the generation of positive growth signals has been well documented (reviewed Berridge et al. (1985); Berridge (1987a)) as has the manipulation of this pathway by chemical and viral agents which influence growth control (Section 1.D.1.3), therefore making this a logical positive signal to examine in the transformed and tumourigenic hybrids.

Several aspects of phosphoinositide metabolism were investigated, not only in the somatic cell hybrids but also in

the normal and tumourigenic parental lines, in order to assess at what stage in the tumourigenic process any changes may have occurred. In addition, an effector of the phosphoinositide second messenger system, protein kinase C, was also examined to assess whether this pathway may have been circumvented, if not deregulated in the tumourigenic hybrids. Protein kinase C, was chosen due to its multifaceted role in co-ordinating cell growth and differentiation (Kikkawa and Nishizuka (1986); Berridge (1987a)).

CHAPTER 2:

GENERAL METHODS

Definition and Maintenance of the Cell System

The human somatic cell hybrids used in this study were derived from the fusion of the tumourigenic D98AH$_2$-clone of the human carcinoma line, HeLa, with normal human diploid fibroblasts. All hybrid lines were developed and generously provided for use in Dr Banyard's Laboratory by both Prof. E.J. Stanbridge (Dept. Microbiology, College of Medicine, University of California, Irvine, California, 92717) and Dr. H.P. Klinger (Dept. Genetics, and R.F. Kennedy Centre, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York, 10461). Information concerning the method of fusion and further characterization of these cell lines are given in Stanbridge (1976); Stanbridge and Wilkinson (1978); Stanbridge and Ceredig (1981); Klinger (1980). In each case, both the original non-tumourigenic cell hybrid and its tumourigenic segregant were provided such that cell lines could be analysed as pairs.

All cell hybrids maintained in tissue culture displayed a transformed phenotype in that they exhibited an indefinite life span *in vitro* (immortalized), grew rapidly in the presence of low serum, and reached high population densities similar to those obtained by the D98AH$_2$ cells (Stanbridge *et al*. (1982), personal observation). The hybrids grew as adherent colonies with a morphology intermediate between their fibroblastic and the epithelial HeLa parents.

Cells representing the parental lines were also analysed, with the original $D98AH_2$ line being supplied by Prof. Stanbridge. The human fibroblast cell line MRC-5 was obtained from Flow Laboratories (Scotland, U.K.) and the A183 primary fibroblast cultures were established from amniocentesis samples supplied by Dr. Flower (Royal Children's Hospital Research Foundation, Victoria, Australia). A list of the cell lines used, and their growth behaviour both *in vitro* and *in vivo* are given in the following Table.

TABLE 2A: Growth Characteristics of the Culture Lines Used

Cell Line	Growth Behaviour *in vitro*	Growth Behaviour *in vivo*
39EC13	transformed	non-tumourigenic
ESH39	transformed	tumourigenic
5E	transformed	non-tumourigenic
5L	transformed	tumourigenic
$Cn_2B_1Col_1$	transformed	non-tumourigenc
5A7mp	transformed	tumourigenic
MRC-5 or A183	normal	non-tumourigenic
$D98AH_2$	transformed	tumourigenic

39EC13, ESH39, 5E, 5L and $D98AH_2$ were supplied by Prof. E.J. Stanbridge; $Cn_2B_1Col_1$ and 5A7mp were from Dr H.P. Klinger.

One of the advantages of this cell system is the genetic stability of the hybrids in culture (Stanbridge *et al.* (1982)). However, all cell lines were routinely tested for tumourigenicity by injection of $5x10^6$ cells subcutaneously into nude mice. Cell lines which produced progressively growing

tumours within 2-3 weeks of innoculation were classified as tumourigenic. Cells were also regularly tested for contamination by mycoplasma using the method of Chen (1977).

All cells were maintained in Dulbecco's Modified Essential Medium (DMEM) [Gibco Laboratories, (U.S.A.)] supplemented with 2mM sodium pyruvate, 2mM L-glutamine and 30mg/L penicillin G and containing either 5% (v/v) fetal calf serum and 5% (v/v) new born calf serum (for the 39EC13, ESH39, 5E and 5L cells) or 10% (v/v) fetal calf serum (for the $Cn_2B_1Col_1$, 5A7mp, $D98AH_2$ and fibroblast lines). The antibiotics neomycin sulphate (50mg/L) and Streptomycin sulfate (50mg/L), used previously in this tissue culture system were omitted due to the reported inhibition of the phosphoinositide pathway by such compounds (Orsulakova et al. (1976; Downes and Michell (1981)). Cells were grown in plastic flasks and dishes of tissue culture quality at 37°C in a 5% CO_2 (in air) humidified atmosphere.

Because all cells are adherent, they were routinely trypsinized before subculture. Briefly, after the medium was removed, cells were washed with warm (37°C) physiologically buffered saline (PBS - see Appendix for formula) and then incubated for 7 minutes with 0.025% (w/v) trypsin in PBS+ 0.02% (w/v) ethylenediaminetetraacetic acid (EDTA) at 37°C. The trypsin was neutralized with an equal volume of medium and the cells collected by centrifugation at 2000g for 5 minutes. The cell pellet was suspended in serum supplemented medium and a proportion of the cells seeded into a new flask. When cell counts were performed 0.2% trypan blue was used to assess cell death.

Stocks of each cell line were stored in liquid nitrogen, the cells being frozen at $1\text{-}2\times10^6$ cell/mL in serum supplemented medium containing 10% (v/v) Dimethylsulphoxide.

CHAPTER 3:

EXAMINATION OF PHOSPHOINOSITIDE METABOLISM IN THE TRANSFORMED AND TUMOURIGENIC HeLa/FIBROBLAST SOMATIC CELL HYBRIDS:

Investigation of possible deregulation of second messenger production.

3.A INTRODUCTION

Receptor driven phosphoinositide hydrolysis is clearly involved in the transduction of growth signals across the plasma membrane (for reviews see Berridge (1987a), Vicentini and Villereal (1986)). Because of this, many studies have examined the operation of the phosphoinositide pathway in cells whose growth regulation is altered, to determine whether an imbalance in this positive growth signal is associated with the generation of the tumourigenic phenotype.

Probably the first correlation between tumourigenesis and the phosphoinositide pathway came from the observations that the tumour-promoting phorbol esters and several structurally unrelated tumour promoters exert many of their pleiotropic effects on cell growth through their ability to activate the enzyme, protein kinase C (Nishizuka (1984); Kikkawa and Nishizuka (1986)). However, as early as 1977, (the time at which protein kinase C was first being characterized (Inoue et al. (1977))) it was found that changes in phosphatidylinositol metabolism correlated with the growth

state of normal and Rous Sarcoma Virus-transformed Japanese quail cells (Diringer and Friis (1977)). Since then it has been shown that many of the viral oncogenes, including ras, ros, scr, fms, fes, sis and the transforming viral gene product polyoma middle T, are capable of influencing the metabolism of the phosphoinositides (see Section 1.D.1.3) and that increased phosphoinositide turnover or alterations in the enzymes associated with phosphoinositide metabolism, are observed in several chemically transformed cell lines (Kuboto et al. (1986); Rillema (1986)).

The majority of information, concerning the involvement of the phosphoinositide pathway in the loss of cellular growth control, concerns the investigation of phosphoinositide metabolism in established cell lines before and after transfection with DNA tumour viruses (eg Jackowski et al. (1986); Fleischman et al. (1986). A number of the oncogene and viral gene (polyoma middle T) products have been reported to have an associated phosphatidylinositol kinase activity in vitro (Kaplan et al. (1986); Whitman et al. (1986)) and it is probable that the observed changes in phosphoinositide metabolism, generated by activated oncogenes, are due to the direct action of these dominantly acting genetic elements upon the enzymes of the phosphoinositide pathway. It is assumed that these changes are associated with the production of a fully tumourigenic state although it is often not clearly defined at which stage in the transition from the normal to tumourigenic phenotype these changes are occurring.

The aim of the present study was to examine the operation of the phosphoinositide pathway in the transformed

and tumourigenic HeLa x fibroblast human somatic cell hybrids on the premise that any noted changes in phosphoinositide metabolism would be associated with the events required for the maintenance, if not the generation, of the tumourigenic as opposed to the transformed state. Moreover, since the expression of the tumourigenic phenotype in the somatic cell hybrid system is associated with the loss of specific genetic elements, presumed to be carrying tumour suppressor genes (Stanbridge (1985)) a perceived change in the phosphoinositide pathway in these cells would not only give an insight into the functioning of these suppressor genes but also indicate that apparently divergent mechanisms for generating tumours *in vivo* (activation of oncogenes *versus* loss of suppressor genes) converge at the one biochemical pathway.

Further incentive to seek a change in the phosphoinositide pathway in the tumourigenic somatic cell hybrids came from a report by Banyard and Tellam (1985) which indicated that intracellular free calcium concentrations in these cells were elevated compared with their transformed partners. Although there are many possible explanations for this rise, it was of interest to determine whether it could be attributed to an elevation in the steady state level of Ins $(1,4,5)P_3$ which has been shown to elevate $[Ca^{2+}]_i$ during signal transduction by liberation of Ca^{2+} from internal stores (Berridge *et al*. (1984)).

In addition, several other changes associated with the assumption of the tumourigenic phenotype might be explained by an alteration in the phosphoinositide signal transduction

pathway. For example, the changes in the cytoskeleton that occur in the tumourigenic hybrids (Der et al. (1981); Gowing et al. (1984)) could be mediated, at least in part, not only by protein phosphorylations generated by protein kinase C (PKC) (Werth et al. (1983); Litchfield and Ball (1986)) but also, by changes in the reported interaction of -actinin with diacylglycerol (Burn et al. (1985)) and profilactin with phosphatidylinositol(4,5)- bisphosphate (PIP_2) (Lassing and Lindberg (1985)). Likewise, the changes in glucose transport in tumourigenic hybrid lines (White et al. (1983)) may be mediated by the phosphorylation of the glucose transporter by PKC (Witters et al. (1985)).

Phosphoinositide metabolism was examined in the transformed and tumourigenic human somatic cell hybrids and the cells representing their parents, by monitoring the incorporation of [^{3}H]-inositol into the total inositol lipid pool in response to continuous serum stimulation. The relative size of the total exchangeable phosphoinositide pool was also determined and the levels of the inositol trisphosphate isomers compared in order to assess the functioning of the phosphoinositide pathway in the transformed and tumourigenic cells.

3.B MATERIALS AND METHODS

For maintenance and definition of cell system see Chapter 2.

3.B.1 LABELLING MEDIUM FOR [^{3}H]-INOSITOL INCORPORATION EXPERIMENTS:

Because the provision of inositol in the medium is essential for the normal growth of some cells in tissue culture (Eagle *et al.* (1960)), it was important to ensure that the lowering of extracellular inositol concentrations, during labelling, did not compromise the growth of the somatic cell hybrids. Two hybrid cell lines (5E and 5L) were grown in either DMEM or the commercially available inositol free medium, Basal Medium Eagles (Flow Laboratories, Scotland, UK) and their growth rates were compared. It was found that both cell lines grew equally well over a 72 hour period with or without inositol in the medium, suggesting that these cells either contain a large intracellular store of free inositol (as suggested by the decay experiments, see Section 3.C.1) or that they can synthesis it *de novo* from glucose (Eisenberg (1967)).

When the amount of label incorporated into cellular membranes after 16 hours incubation was related to the concentration of *myo*-[2-^{3}H] inositol (603 GBq/mmol; 1mCi/mL: Amersham Int., Australia) in the medium, it was found that below a concentration of 185kBq/mL (5μCi/mL), the level of incorporation was dependent on label concentration but that increasing the concentration above this level served little advantage. Therefore, a concentration of [^{3}H]-inositol of 185-277.5kBq/mL (5-7.5μCi/mL) was routinely used.

3.B.2 DECAY EXPERIMENTS:

The procedure used was continually modified during the course of these experiments (see results section 3.C.1). The final protocol was as follows:-

Cells, plated at 1-5 x 10^5 cells/dish in 35mm diameter plastic petri dishes, were grown for 24 hours in supplemented DMEM before being washed with one mL phosphate buffered saline (PBS - see Appendix for composition) and radio-labelled for 24 hours in Basal Medium Eagle (BME) containing 7.5μCi/mL myo-[2-^{3}H] inositol (16.3 Ci/mmol; 603 GBq/mmol). After the labelling period, cells were washed twice with supplemented DMEM and then re-fed with 1 mL supplemented DMEM or processed as time zero. At appropriate times the medium was removed and cells washed twice with PBS then precipitated with 1 mL ice-cold 10% (w/v) trichloroacetic acid (TCA). Dishes were left on ice for 5 min, washed with a second mL of TCA then 1 mL H_2O. These 3mL were combined and assayed for the presence of inositol trisphosphate ($InsP_3$) as outlined below.

The TCA precipitate, which contained the inositol lipids, was scraped into 1mL H_2O and the plate rinsed with a second mL H_2O. A sample from the combined 2mL was taken for protein determination using the modified Lowry method (see 3.B.9 of this section). 1.5mL of the TCA precipitate were added to 1.5mL chloroform, the solution vortexed and allowed to phase separate under gravity. The lower phase was collected and the upper phase washed with 1.5mL chloroform. The lower phases were then combined and deacylated as in Downes and Michell (1981). Briefly, the 3mL chloroform extract was added to 0.6mL of

methanol and 0.6mL of 1N NaOH in methanol:H_2O (19:1(v:v)) (this solution was made by solubilizing NaOH in H_2O before addition of methanol and was stored in a dark bottle). The mixture was vortexed 30 sec maximum speed, and left at room temperature for 20 min., after which 6.6mL of chloroform:methanol:H_2O (10:6:6 (v/v/v)) were added, the solution vortexed and allowed to separate. The aqueous phase was then collected for separation on dowex ion exchange columns.

The Dowex used for the separation of both the inositol phosphates and the deacylated lipids, was bought as the 200-400 mesh chloride form (Sigma Chemical Co. USA) and was converted to the formate form by equilibrating the resin with 20 volumes 1M NaOH then 20 volumes 1M formic acid. Originally, each column was converted separately and re-used but later the formate form was made in bulk and columns repacked between experiments to improve reproducibility. (Bio.Rad now market an exchange resin in the formate form which is also reported to give good separations (personal communication).)

Tests of efficiency of the dowex resin packed as 1cm diam. x 1cm columns were performed by chromatographing standard $InsP_3$ (from Sigma Chemical Co. USA) or deacylated phosphatidylinositol(4,5)bisphosphate (Sigma Chemical Co. USA) and assaying the elution fractions for inorganic phosphate using the malachite green assay (see 3.B.9). To monitor the functionality of the columns during each experiment, a non-saturating concentration of ATP (0.5μmol.) was loaded with each sample and the absorbance of the elutant measured at 260nM.

For the $InsP_3$ separation, 3mL of TCA extracts were extracted once with one volume of ether, to remove much of the TCA and then neutralized by addition of 1.5mL of 1M Hepes, pH7.0. The sample was loaded onto the column with ATP and batchwise eluted according to Lapetina *et al*. (1985):-

Step	Volume (mL)	Eluting Buffer	Species eluted
(i)	2 x 6mL	H_2O	inositol
(ii)	2 x 6mL	5mM sodium tetraborate, 60mM ammonium formate	glycero-phosphoinositol
(iii)	2 x 6mL	0.2M ammonium formate; 0.1M formate	InsP
(iv)	3 x 6mL	0.4M ammonium formate; 0.1M formate	$InsP_2$
(v)	3 x 4mL	1.0M ammonium formate; 0.1M formate	$InsP_3$
(vi)	1 x 8mL	1.5M ammonium formate; 0.1M formate	$InsP_4$?

This last step (vi) was originally included to wash the columns. However, the radioactivity associated with this fraction is probably the, since discovered, inositol tetrakisphosphate, $InsP_4$ (Batty *et al*. (1985)). Fractions (v) and (vi) were freeze dried for 48 hours and then resolubilized in 1mL H_2O. Ten millilitres of an aqueous scintillant containing 66%(v/v) xylene, 33%(v/v) triton X114 and 0.5%(w/v) 2,5 diphenyloxazole (PPO) were added and the

radioactivity assessed by liquid scintillation counting. Results were expressed per mg protein.

For the deacylated lipid samples, the aqueous phase was made up to 4.5mL with H_2O and 0.5mL of 50mM disodium tetraborate was added such that the final concentration of disodium tetraborate was 5mM. Samples were then loaded onto Dowex with ATP and eluted as in Downes and Michell (1981) as follows:-

Step	Volume (mL)	Eluting buffer	Species eluted
(i)	4x5	5mM disodium tetraborate; 0.18M ammonium formate	everything except glycero-phosphoinositol(4) phosphate and glycerophospho-inositol(4,5)-bisphosphate
(ii)	3x6	0.3M ammonium formate; 0.1M formate	glycerophosphoinositol -(4)phosphate
(iii)	4x5	0.75M ammonium formate; 0.1M formate	glycerophosphoinositol -(4,5)bisphosphate
(iv)	3x5	1.2M ammonium formate; 0.1M formate	to clean the column.

Fractions (iii) and (iv) were freeze dried and counted as above.

3.B.3 RE-APPRAISAL OF LABELLING MEDIUM:

It was noted from the decay experiments that the extent of incorporation of [^{3}H]-inositol into cellular membranes was very low with approximately 1% of externally added label being incorporated into 10^5 cells within 24 hours. As reports had indicated that, in some cell types glucose competes with inositol for entry into the cell (Green and Lattimer (1982); Gillon and Hawthorne (1983)), the effect of glucose on inositol incorporation in the HeLa cell system was tested. Preliminary experiments with HeLa spinner cultures suggested that 5mM glucose in the medium did depress inositol incorporation, although the effect was not significant in the hybrids. Nevertheless, since cell growth (as assessed by doubling time) was unaffected, irrespective of carbon source (glucose versus fructose), in the four cell lines examined, a minimal essential medium free of inositol and glucose (MEM-G) was used for subsequent labelling experiments. (See Appendix for composition.)

3.B.4 KINETICS OF INCORPORATION OF [^{3}H]-INOSITOL INTO CELLULAR MEMBRANES:

Cell pairs were plated as replicates in 35mm diameter plastic petri dishes at 1 x 10^5 cells/dish in Supplemented DMEM. After 24-48 hours the medium was aspirated and the cells rinsed with 2mL of the supplemented labelling medium, MEM-G (composition given in Appendix).

The dishes were then either (a) fed with supplemented MEM-G and used to obtain cell numbers at the

beginning and the end of the timecourse or (b) labelled with 1mL of a stock solution of supplemented MEM-G containing 185kBq/mL (5μCi/mL) of [2-^{3}H]-inositol (603 GBq/mmol). At various timepoints the medium was removed from duplicates and the dishes washed with ice-cold PBS. One mL ice-cold 10% (w/v) trichloroacetic acid (TCA) was then added. After 5 minutes on ice, the plates were washed with another 1mL 10% (w/v) TCA followed by 1mL H_2O. After removal of the H_2O wash, the lipids were extracted from the TCA precipitate by addition of 0.8mL of chloroform:methanol:conc.HCl (100:200:2(v:v:v)). This lipid extract was transferred to plastic scintillation vials and, after combination with a second 0.8mL chloroform:methanol: HCl rinse, 10mL of organic scintillation fluid (100% (v/v) xylene, 0.5% (w/v) PPO) were added for liquid scintillation counting.

Therefore, the inositol containing phospholipids were defined as that proportion of radioactivity which was both precipitated by 10% TCA and subsequently extracted by chloroform:methanol: conc.HCl (100:200:2(v:v:v)). This assumes that all membrane phospholipids are precipitated by TCA and recognises that those phosphoinositides covalently bound to protein may not be extracted from the precipitate. It has been documented that phosphatidylinositol acts as an anchoring lipid for several cell surface proteins such as Thy-1 and acetylcholinesterase (Low et al. (1986); Cross (1987)). However, since the orientation of the inositol head group is facing outside the cell, these inositol lipids are unlikely to be involved in the primary signal transduction event.

3.B.5 RELATIVE SIZE OF THE TOTAL EXCHANGEABLE INOSITOL LIPID STEADY STATE POOLS:

These experiments were performed in the same way as the kinetic studies except that six replicates/cell line were processed when the system had reached equilibrium, at 45-46 hours. Concurrent determinations of cell number were performed on five dishes.

3.B.6 CELL SURFACE AREA MEASUREMENTS:

After examining several indices of membrane surface area, the measurement of total membrane palmitate was considered the most suitable method for these studies. Cells were labelled for 45-46 hours to equilibrium with 3.7kBq/mL (0.1μCi/mL) of [1-^{14}C]-palmitate 1.85GBq/mmol; 50mCi/mmol) (from New England Nuclear, USA) in 1mL of supplemented MEM-G and then processed as in the inositol incorporation experiments.

3.B.7 THIN LAYER CHROMATOGRAPHY OF INOSITOL LIPIDS:

Cells were grown on 35x9mm glass rectangular coverslips in supplement DMEM and labelled for 48 hours in supplemented MEM-G containing 185kBq/mL (5μCi /mL) [2-^{3}H]-myo inositol. After labelling, the slips were washed X 6 in PBS at room temperature and the cellular lipids extracted in a manner similar to that outlined by Billah and Lapetina (1982) and modified by Damian Myers (personal communication). Cells were incubated for 1 hour at room temperature in 4 mL of $CHCl_3$:MeOH:0.83M HCl (9:7:2(v:v:v)) under N_2. The chloroform extract was transferred to a clean tube and 1.12 mL

of 2M KCL was added. The solutions were vortexed for 5 seconds at maximum speed and centrifuged at 5000 g for 5 min in a bench top centrifuge to facilitate phase separation. The lower phase was collected and the upper aqueous phase washed with 2.2mL of $CHCl_2$:MeOH:0.83M HCl (9:7:2(v:v:v)). The lower phases were combined and after addition of $\cong 5\mu g$ of bovine brain phosphoinositides (Sigma Chemical Co. USA) were dried down under N_2. Samples were resuspended in $50\mu L$ of $CHCl_3$:MeOH: 0.83N HCl (9:7:2(v:v:v)), $30\mu L$ of which were applied to a 10x20 cm Silica 60^{F254} thin layer chromatography plate (Merck; Germany) which had been previously soaked overnight in 1% (w/v) potassium oxolate; 2mM ethylenediaminetetraacetic acid (EDTA) in MeOH:H_2O (2:3(v:v)) and heat activated in a dry oven at 100° for 1 hour prior to use. Samples were then developed with authentic standards (phosphatidylinositol (PI); phosphatidylinositol(4)phosphate (PIP); phosphatidylinositol-(4,5)bisphosphate (PIP_2) and phosphatidate - all from Sigma Chemical Co. USA) in a paper lined chromatography tank using the solvent system $CHCl_3$:Acetone:MeOH:acetic acid: H_2O (40:15:13:12:8 (v:v:v:v:v)) as in Jolles, et al. (1981). The plates were air dried and the phospholipids visualized with iodine vapour. The appropriate spots were scraped from the plate into scintillation vials. After combination with 0.5mL H_2O and 0.5mL MeOH, 10 mL of aqueous scintillant, (composition outlined previously) was added and radioactivity determined by liquid scintillation counting.

3.B.8 HIGH PERFORMANCE LIQUID CHROMATOGRAPH OF INOSITOL PHOSPHATES:

Cells grown in 75cm^2 tissue culture flasks were labelled for 72 hours in supplemented MEM-G containing 185kBq/mL (5μCi/mL) [2-^{3}H]-myo inositol. At the end of the labelling period the medium was rapidly removed and the cells washed with 1x50mL normal saline (4°C). Cells were lysed with 10mL of ice-cold 10% (w/v) TCA and left on ice for 10 min. After removal of the TCA the flasks were washed with a further 5mL of 10% (w/v) TCA and the combined TCA solutions were extracted x6 with ether (analytical grade not anaesthetic quality). The ether was removed by bubbling N_2 through the solution and the samples were neutralized with 1M Trizma Base. 0.2mM mannitol was added and the samples freeze dried, after which they were stored at -20°C prior to use. Cell number determinations were made from flasks grown in parallel in unlabelled MEM-G for the 72 hour period.

Inositol phosphates were separated by anion exchange chromatography using a Partisil SAX column from Activon (USA) and a Waters high performance liquid chromatography (HPLC) system. The buffer system used was H_2O (buffer A) and 1.7M NH_4 formate, pH 3.7 with orthophosphoric acid (buffer B) as in Batty et al. (1985). Buffers were filtered (0.45 μm pore size; Millipore HV) and de-gassed and the column washed with buffer B at the beginning of each chromatography session. Before loading the sample the column was equilibrated with 1% buffer B to minimize fluxes due to a change in pH at the onset of the gradient. Samples (500-800μL) which had been

resolubilized in H_2O were loaded onto the column with 10μM ATP/ADP and AMP and eluted with a linear gradient of NH_4 formate over 50 min. at 1mL/min as outlined below. Elutions were monitored at 254nm and fractions collected every 30 second from minute 3 to 55 directly into scintillation vials.

Time (min)	Flow (mL/min)	% A	% B	Curve*
0	1	99	1	6
5	1	99	1	6
55	1	12	88	6
60	1	12	88	6

* (Designates a linear gradient on the Waters' system)

Six mL of standard aqueous scintillant were added to fractions corresponding to minute 3-30 and the high performance commercially available scintillant, Aquasol (NEN-Du Pont, USA) was used for fractions 30.5-55 min. 500μL of H_2O was added to each vial to prevent clouding of the scintillant due to the presence of high salt. Radioactivity was assessed by liquid scintillation counting.

3.B.9 PROTEIN AND INORGANIC PHOSPHATE DETERMINATIONS:

Protein determinations of TCA precipitates of cellular material were performed using a modified Lowry Method as outlined originally in Lowry et al. (1951).

Briefly, TCA precipitates were collected by centrifugation at 10 000g, 15 min. and resuspended in 100μL 1M NaOH. After addition of 1mL of solution C (prepared just prior to use by the combination of 2 volumes of 1% (w/v) Cu_2SO_4; 1% (w/v) NaK tartrate with 100 volumes of 2% (w/v) Na_2CO_3), solutions were incubated at room temperature (RT)

for 10 min. 10μL Folin-Ciocalteu reagent (BDH Chemicals Pty. Ltd. Aust.), diluted 1:3(v:v) was added with thorough mixing and the solutions incubated for a further 30 min. at RT before measurement of spectrophotometric absorption at 750nm. Protein standards were concurrently prepared in the range 0-10μg using 1mg/mL bovine serum albumin (BSA fraction 5) (Armour Pharmaceutical Co. UK) in 1M NaOH.

Inorganic phosphate determinations of Dowex column elutions were performed according to the method of Stull and Buss (1977); Itaya and Ui (1966). Fifty microlitres (50μL) of column elutions were transferred to Pyrex tubes which had been washed (as was all glassware) in 6M HCl. Twenty-five microlitres (25μL) of 10% (w/v) $MgNO_3.6H_2H$ in 95% (v/v) absolute ethanol was added and the samples ashed to liberate inorganic phosphate. This was performed in a fume hood and achieved by gentle hating, over a blue gas flame, until no further brown fumes had been emitted and the sample had formed a grey-white precipitate (note that black precipitates give spurious results). The precipitate was dissolved in 750μL 1.2M HCl and 250μL malachite green solution added. This latter solution, made just prior to use, contained 1 volume 10% (w/v) $(NH_4)_6\ MoO_{24}.4H_2O$ in 4M HCl (molybdate dissolved in H_2O prior to addition of acid) with 3 volumes 0.2% (w/v) malachite green (Sigma Chemical Co., USA) and was filtered before use. Absorbance at 660nm was monitored immediately after addition of malachite green solution and readings were taken when absorbance had reached a plateau. Standard phosphate measurements in the 0-50nmol range were concurrently performed with 1mM KH_2PO_4.

3.C RESULTS AND DISCUSSION

3.C.1. DECAY EXPERIMENTS:

Originally, it was planned to examine the metabolism of the phosphoinositides in the different cell types after long term labelling with [^{3}H]-inositol by monitoring the serum-stimulated decay of phosphatidylinositol bisphosphate (PIP_2) from the membrane and the subsequent production of inositol trisphosphate(s) ($InsP_3$) after transfer of the cells to unlabelled medium.

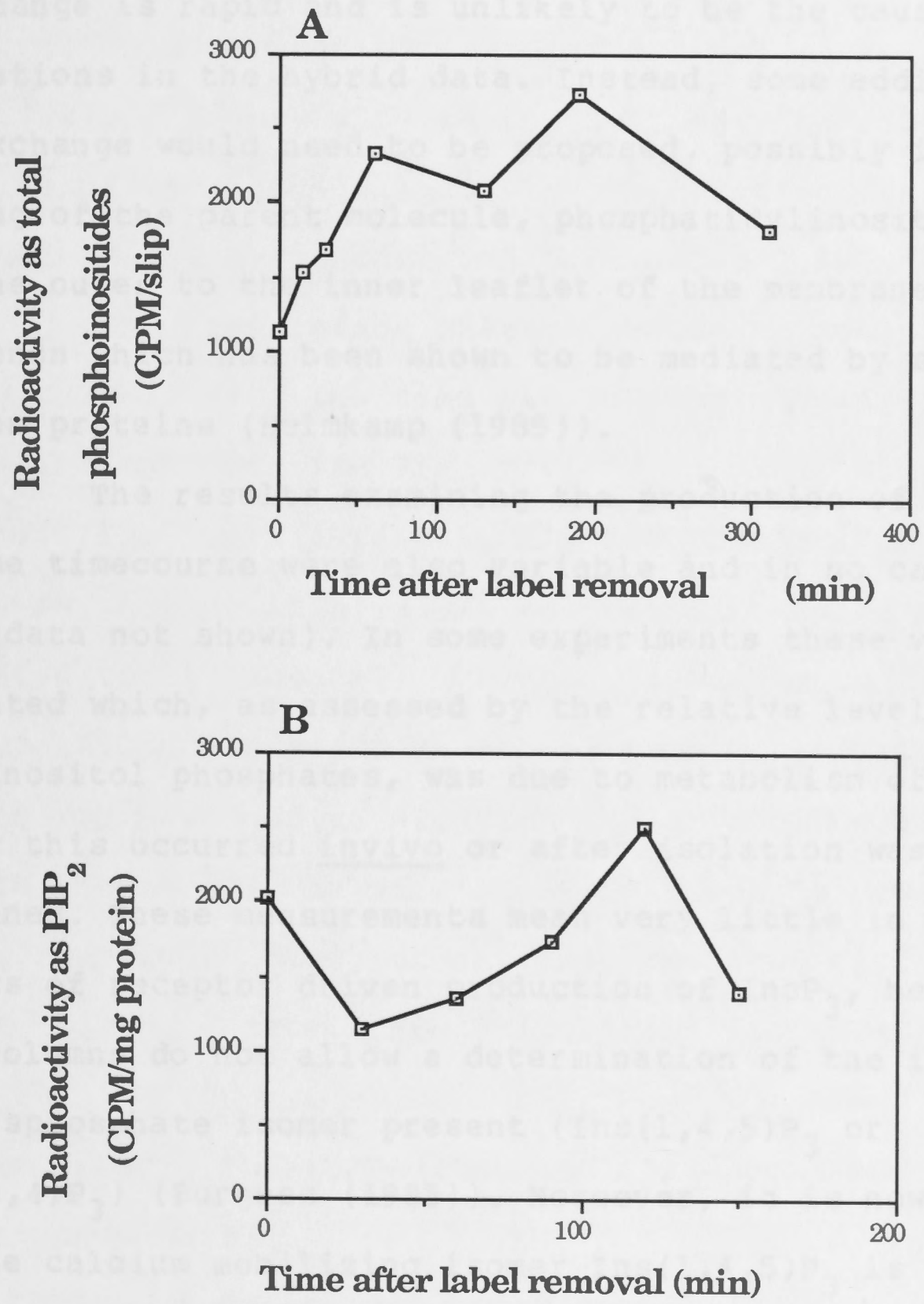
Preliminary studies, in which the total inositol lipid pools were monitored (by measuring the trichloroacetic acid precipitable radioactivity at different timepoints), indicated that the size of the labelled phosphoinositide pool did not decay within the first 30 min of label removal and oscillated around a fixed value for the next four hours see figure 3.1. Addition of 10mM LiCl in to unlabelled medium to prevent re-synthesis of the phosphoinositides from the metabolized inositol phosphates did not alter the pattern of oscillations. It was assumed, therefore, that there was a large intracellular pool of free inositol (reported by others Jackowski *et al*. (1986) and so a single measurement was made after the removal of the radiolabelled medium but no further measurements were made until the cells had spent 1 hour in an unlabelled medium in an attempt to deplete the intracellular store of free inositol.

When the decay of the individual phospholipid PIP_2 was examined, oscillations were also found and despite numerous

experiments, no reliable measurement for membrane decay could be obtained (see figure 3.1). The pattern of oscillations varied between experiments, possibly due to the asynchrony of the cell population which would not be reproducible from one set of experiments to another. A number of controls were performed to assess the reproducibility of the experimental techniques but none could compensate for the fluctuations. The efficiency of the deacylation procedure was checked using standard PIP_2, the subsequent partitioning into the aqueous phase of which was monitored using inorganic phosphate determinations. The functionality of the Dowex columns was assessed by monitoring the elution of standard $InsP_3$ and deacylated PIP_2 and during each experiment, by monitoring the elution of ATP. Neither of these factors were responsible for the oscillations. The lipid extraction procedure was also not the source of variation as the fluctuations between timepoints seen in the total chloroform extract, did not mirror those of the isolated PIP_2. It is possible, given the report by Gumber and Lowenstein (1986) indicating that, in a mixture of chloroform, methanol and H_2O, phosphatidylinositol phosphate (PIP), PIP_2 and phosphatidic acid undergo non-enzymic phosphorylations in the presence of bivalent metal ions, that variations in the present study may have occurred between the extraction and deacylation steps. However, in order for this to explain the observed variations, it would also have to be assumed that this reaction was not uniform between timepoints.

The observed oscillations in PIP_2 levels in the hybrids could be explained from a biological viewpoint by the

FIGURE 3.1
DECAY OF THE PHOSPHOINOSITIDES



Results demonstrate the level of [^{3} H]-inositol remaining in the total phosphoinositide pool (graph A) or as phosphatidylinositol bisphosphate (PIP_2) (graph B) after removal of the label.Results represent single separate experiments.

existence of multiple metabolic pools of PIP_2 which interchange slowly with other inositol containing lipids. A rapid interchange between the phosphoinosides mediated by phosphorylation-dephosphorylation reactions has been well documented (futile cycles see diag. 1.3). However, this interchange is rapid and is unlikely to be the cause of the fluctuations in the hybrid data. Instead, some additional long term exchange would need to be proposed, possibly involving the flipping of the parent molecule, phosphatidylinositol (PI), from the outer to the inner leaflet of the membrane, a phenomenon which has been shown to be mediated by specific transfer proteins (Helmkamp (1985)).

The results examining the production of $InsP_3$ over the same timecourse were also variable and in no case showed a decay (data not shown). In some experiments these values oscillated which, as assessed by the relative levels of the other inositol phosphates, was due to metabolism of $InsP_3$. Whether this occurred <u>invivo</u> or after isolation was not determined. These measurements mean very little in terms of the dynamics of receptor driven production of $InsP_3$, because the dowex columns do not allow a determination of the identity of the trisphosphate isomer present ($Ins(1,4,5)P_3$ or $Ins(1,3,4)P_3$) (Burgess (1985)). Moreover, it is now apparent that the calcium mobilizing isomer $Ins(1,4,5)P_3$ is not only metabolized within a minute of its production but also that its formation may not be continuous, even in the constant presence of an agonist (Irvine <u>et al</u>. (1985), Burgess <u>et al</u>. (1985)). Therefore, the measurements being made during the hybrid study

more accurately represent the steady-state levels of IP_3 over that time period. The fact that the levels of radiolabelled IP_3 changed very little over the 3 hours, suggested either that the pool of PIP_2 which is cleaved to produce IP_3 has approximately the same specific activity over that entire period, or that the metabolism of the inositol trisphosphate ($Ins(1,3,4)P_3$ is very slow in the hybrids. The size of the steady state $Ins(1,4,5)P_3$ pool in the different cell types is important and will be addressed in section 3.1.6.

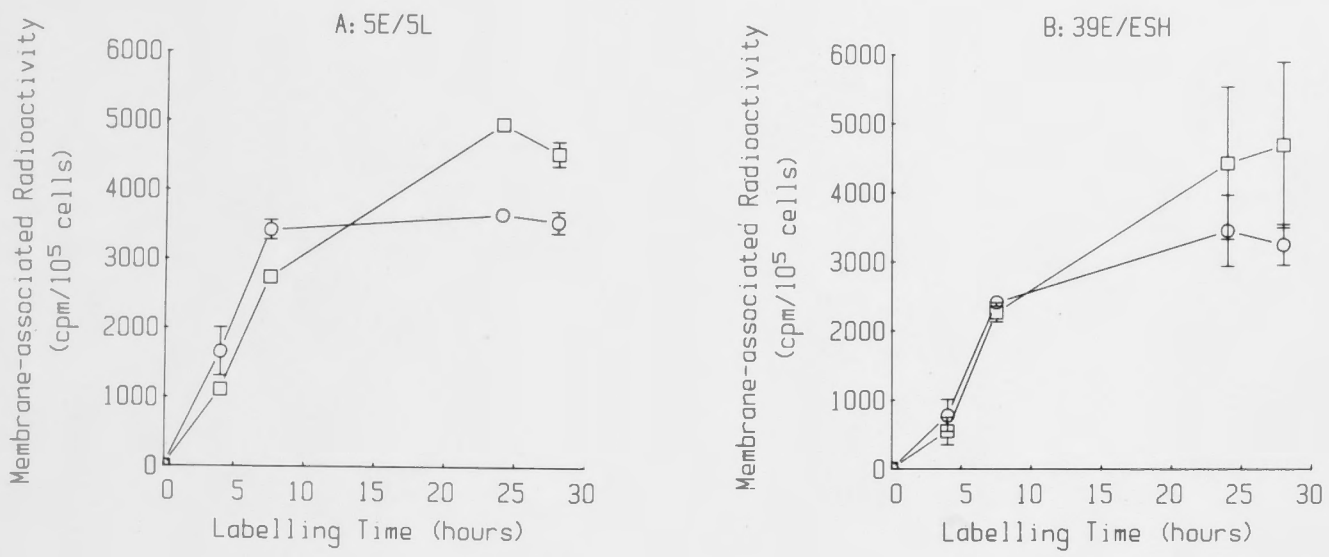
The observation that there was no notable decay of the PIP_2 levels over the time period examined may indicate that the receptor driven PIP_2 pool is small in comparison to the total PIP_2 pool. Alternatively the phosphoinositide pathway may not be involved in the maintenance of serum stimulated continuous cell growth in the somatic cell hybrids (in support of this see Tones et al. (1988)). It was apparent that the fluctuations in experimental data did not permit reliable interpretation of PIP_2 decay rate. To complement the data an associated study of the incorporation of [^{3}H]-myo inositol into the total phosphoinositide pool was undertaken.

3.C.2 KINETICS OF INCORPORATION OF [^{3}H]-INOSITOL INTO CELLULAR MEMBRANES:

The rate of incorporation of [^{3}H]-inositol into the cell membranes of two cell hybrid pairs and the cell lines representing their parents were examined and composite graphs of their incorporation kinetics are depicted in figure 3.2. A comparison of the parental cell lines was included as a

FIGURE 3.2

KINETICS OF INCORPORATION OF INOSITOL INTO THE PHOSPHOINOSITIDES



The kinetics of incorporation of [^{3}H]-inositol into the total phosphoinositide pools of the non-tumourigenic (□) and tumourigenic (○) cells were assessed over the time periods indicated. Panels A, B, and D pertain to hybrid pairs and panel C concerns the parental lines. Panels A, C and D depict results from a single typical experiment. Panel B is a composite of two experiments. The bars denote the range of each point. If no bar is seen, its dimensions are exceeded by that of the point symbol.

potential means of assessing whether a failure to note a difference between the transformed and tumourigenic hybrids may be due to the alteration having already occurred during the transition from the normal to the transformed state.

From figure 3.2, it can be seen that all the cell lines reached equilibrium by approximately 30 hrs. This timecourse seemed slow, particularly considering a report in freshly isolated hepatocytes (Thomas et al. (1984)) and islets of Langerhans (Montague et al. (1985)) which indicated that the incorporation of ^{3}H into inositol lipids had reached a plateau by 90 min. It was of concern that even in the continuous presence of serum, the inositol head group of the phosphonositides was not being readily metabolized in the tissue culture lines. However, since then it has been confirmed by others that many cells in culture, including pancreatic islets (Biden et al. (1987a)) and NIH 3T3 cells (Fleischman et al. (1986)) also require over 24 hours labelling with [^{3}H]-inositol to reach isotopic equilibrium. Since it has been established that the mitogenic stimulation of many of these cell lines does involve PIP_2 breakdown, a plausible reason for the slow kinetics is that the receptor coupled phosphoinositide pool is small in comparison to that of the total phosphoinositides (eg Creba et al. (1983)) and that the turnover of this minor receptor-coupled subclass of phosphoinositides is the primary means by which the headgroup of these lipids is exchanged. If this assumption, (that the receptor-coupled phosphoinositide pool is small and is solely responsible for phosphoinositide metabolism) is true then the

inferred rate of decay of the entire phosphoinositide pool in the presence of serum would reflect that of receptor-coupled PIP_2.

A comparison between the hybrid cells (panels A & B) of the kinetics of incorporation indicated that there was very little difference in the kinetics of incorporation between the transformed and tumourigenic cell lines. Moreover, a detailed study of the initial rates of incorporation in the 39EC13 and ESH39 pair (panel D) indicated that there was no difference at all between cell types.

It could be inferred indirectly from this that the rate of decay of the phosphoinositides would also be very similar, an assumption which would be strengthened by conformation of the steady state pool sizes (see next section).

Examination of the kinetics of incorporation of the parental lines $D98AH_2$ and MRC-5 (panel C) indicated, somewhat surprisingly, that the fibroblast line greatly exceeded the tumourigenic HeLa line in both the initial rate of inositol incorporation and the steady state membrane level. However, the factor by which both parameters differed was approximately the same, suggesting that if the values were normalised against some parameter other than cell number then the incorporation kinetics for these lines could also be superimposed, implying that the rate of breakdown in the normal and tumourigenic cells is also the same. It is recognized that in the case of the parental lines, the comparison is being made between cells of vastly different morphology and therefore any alterations in phosphoinositide metabolism generated by an alteration in

growth regulation may be obscured by differences in cell type. However, it has been reported that steady state levels of PIP_2 and diacyglycerol were very similar in non-transformed cells originating from different tissues and species. (Fleischman et al. (1986)).

It could be argued that the failure to note an appreciable difference in the metabolism of the phosphoinositide pool, in any of the cells examined, may be because the measurement of the total inositol lipid pool is too insensitive to reveal the types of specific changes in phosphoinositide metabolism, which are associated with its role in signal transduction. In defence of this, however, studies examining phosphoinositide metabolism in normal and transformed or tumourigenic cells have reported substantial alteration in the levels of phosphatidylinositol (PI) (Kubota et al. (1986) and by virtue of the large contribution PI makes to the inositol containing lipids (Jackowski et al. (1986)) such changes would be detected in the present study of total phosphoinositides. In addition, more direct evidence that changes in the levels of the PIP_2 pool are reflected by total phosphoinositide measurements in the hybrids is given in section 3.C.6.

3.C.3 RELATIVE SIZE OF THE TOTAL EXCHANGEABLE INOSITOL LIPID STEADY STATE POOLS:

As a number of studies have examined the steady state levels of the phosphoinositides in transformed and un-transformed cells (Fleischman et al. (1986); Jackowski et

al. (1986)) it was considered appropriate to verify the relative size of such pools in the different somatic cell hybrids (see table 3.1). Six replicates were processed for each cell line after extensive labelling (45 hours). Statistical analysis of intra as well as inter experimental variation was obtained after consultation with Mr R. Cunningham (Statistics Department, Australian National University, Canberra, 2601, Australia). Because the ratio of membrane inositol content/10^5 cells of the non-tumourigenic to tumourigenic cells was examined, data was converted to logorithmic (log.) values before assessment, in order to approximate a normal distribution. All statistical analysis was performed on the log. transformed data, which is why, after conversion back to scalars, confidence limits were presented rather than standard error values.

It was found that there was no significant difference between the pool size of the hybrid pairs 5E and 5L or 39ECl3 and ESH39, indicating that, as a generalized phenomena, the assumption of the tumourigenic phenotype is not accompanied by an alteration in total phosphoinositide levels in the somatic cell hybrid system. However, there was a highly significant difference between the pool size of the $Cn_2B_1Col_1$ and 5A7mp hybrids and both fibroblast lines were significantly different from the $D98AH_2$ HeLa derivative. However, the kinetic studies with the $D98AH_2$ and MRC-5 lines (Fig 3.2) indicated that an increased incorporation rate in the fibroblasts was sufficient to explain the differences in total pool size. This elevated incorporation rate might have been due

TABLE 3.1

RELATIVE STEADY STATE PHOSPHOINOSITIDE LEVELS PER CELL

Cell pair	Ratio of non-tumourigenic/tumourigenic steady state phosphoinositide pools		
	A	B	C
5E/5L	**0.93**	(0.81 , 1.07)	[5]
39EC13/ESH39	**1.11**	(1.00 , 1.23)	[5]
$Cn_2B_1Col_1$/5A7mp	**1.14****	(1.09 , 1.21)	[3]
A183/D98AH_2	**1.70***	(1.55 , 1.87)	[2]
MRC-5/D98AH_2	**2.50***	(2.04 , 3.05)	[2]

Non-tumourigenic and tumourigenic cells were labelled for an extended period with [^{3}H]-inositol (as in 3.B.5) and determinations of the steady state levels of total exhangable phosphoinositides per cell were made concurrently within a cell pair. Mean ratios of non-tumourigenic to tumourigenic steady state pools are presented (column A) with 95% confidence intervals (column B) and number of experiments (column C). Statistical analysis was performed as outlined in Section 3.C.3. A two-sided Students t-test was used to assess significance *$p<0.05$, **$p<0.01$.

to an alteration in the activity of enzymes associated with the incorporation of the inositol head group but it might also have resulted from a physical dissimilarity between the cells. Because there is a perceptible size difference between the fibroblast and D98AH$_2$ cells, a measure of cell surface areas was made in order to assess whether disparity in the steady state pool sizes could be accounted for in this way.

3.C.4. RELATIVE CELL SURFACE AREAS OF THE CELL PAIRS:

Studies by Dr D. McKinnon (personal communication) using electron microscopy have indicated that the plasma membrane of the tumourigenic hybrids has many more microvilli than that of their transformed pairs, which would render misleading standard cell size measurements such as cell diameter. Therefore, what was required was a suitable index of membrane surface area which was both evenly distributed in the membrane and unlikely to be modified in its distribution by the assumption of the tumourigenic phenotype.

Palmitate was chosen as the parameter to be measured (see details in methods) since it is universally distributed in membranes and literature searches failed to find a correlation between an alteration in this molecule and tumourigenesis. Another advantage of this parameter was that it could be measured using an experimental protocol very similar to that used for the inositol lipids, therefore providing a suitable experimental control.

Preliminary kinetic experiments were performed and it was found that membrane palmitate levels, like those of

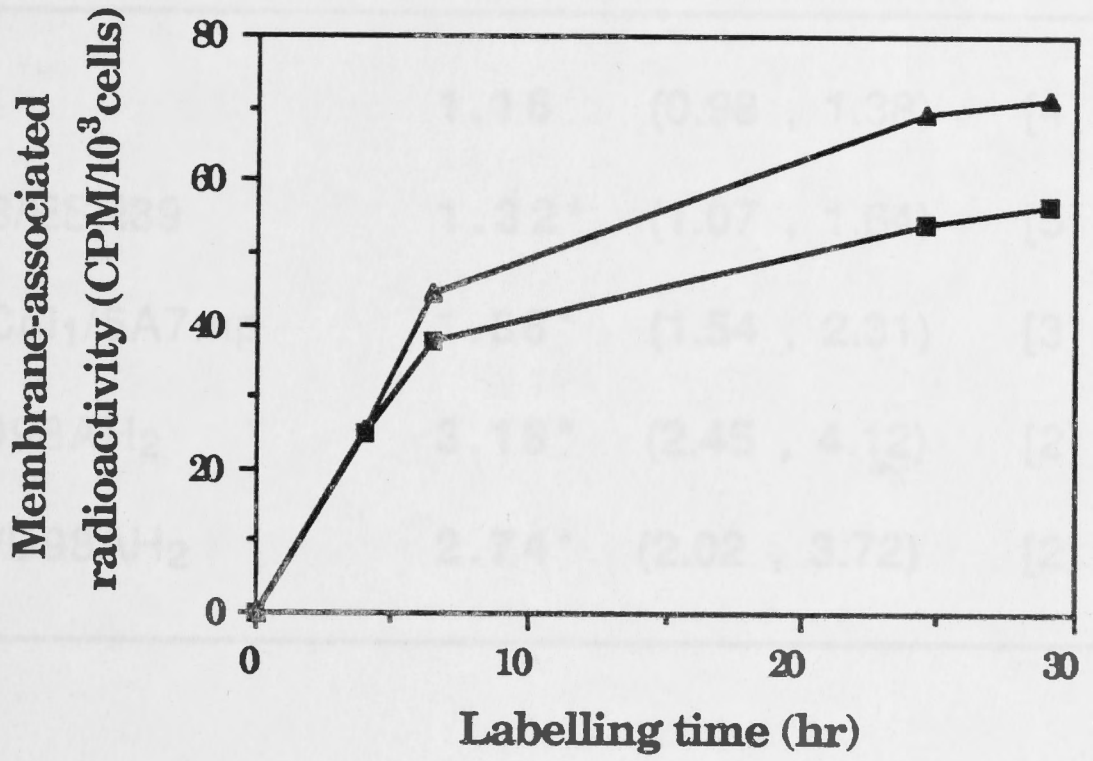
inositol, had reached equilibrium by 30 hours (fig. 3.3). Originally, dual label experiments were performed in order to obtain a ratio of membrane inositol to palmitate from the one dish of cells. However, it was found that, due to the quenching effects of the extraction system, the ^{14}C energy profile was shifted so far to the left that it completely eclipsed the ^{3}H profile, making the distinction of the two isotopes impossible. Therefore, relative palmitate levels were determined in separate experiments which, as discussed later, may have some bearing on the interpretation of results.

Relative palmitate values are given in table 3.2. The confidence intervals and statistical assessment associated with each measurement were obtained in the same way as those for the inositol values. It was found that all non-tumourigenic lines had, on average, more membrane area than their tumourigenic partners but that, in the case of the 5E/5L pair, this difference was not significant. This finding was surprising considering that it was the tumourigenic cell lines which showed a greater number of membrane protrusions but it was also interesting in the context of the inositol data.

Also tabulated are relative total cell protein values. These values were obtained by Dr Gowing and used as an index of cell size in her study on the actin content of the cell hybrids (Gowing et al. (1984)). They are presented here with her permission, to illustrate that the interpretation placed on the relative inositol values may vary, depending on the selection of the parameter by which they are adjusted.

FIGURE 3.3

KINETICS OF INCORPORATION OF PALMITATE INTO CELLULAR MEMBRANES



Results represent a typical experiment monitoring incorporation of [^{14} C]-palmitate into cellular membranes (as outlined in 3.B.6) in transformed (■) and tumourigenic (▲) hybrids. Each point represents duplicate determinations.

TABLE 3.2

RELATIVE CELL SIZE MEASUREMENTS

Cell pair	Ratio of non-tumourigenic/tumourigenic membrane palmitate			total cell protein
	A	B	C	D
5E/5L	**1.16**	(0.98 , 1.38)	[4]	1.00
39EC13/ESH39	**1.32***	(1.07 , 1.64)	[5]	0.99
$Cn_2B_1Col_1$/5A7mp	**1.88***	(1.54 , 2.31)	[3]	0.82
A183/D98AH_2	**3.18***	(2.45 , 4.12)	[2]	nd
MRC-5/D98AH_2	**2.74***	(2.02 , 3.72)	[2]	1.42

Relative membrane surface areas for the non-tumourigenic and tumourigenic cell pairs were obtained in the present study by comparing the steady state levels of cellular palmitate (see 3.B.6). Ratios of the non-tumourigenic to tumourigenic cells are presented (column A) with 95% confidence intervals (column B) and number of experiments (column C). Statistical analysis was performed as outlined in Section 3.C.3. A two-sided Students t-test was used to assess significance *$p<0.05$. Data for relative total cellular protein (column D) was obtained by Dr L Gowing.

3.C.5 RELATIVE PHOSPHOINOSITIDE STEADY STATE POOL SIZES CORRECTED FOR CELL SURFACE AREA:

When the relative inositol values were corrected for membrane palmitate content, it was found that the ratio of the inositol content per unit membrane of the non-tumourigenic to tumourigenic cells (table 3.3) was less than unity in each case. This would imply that there is an association between the assumption of the tumourigenic phenotype and an elevated level of inositol lipids per unit membrane. However, from confidence intervals obtained by taking the square root of the sum of the variance of the separate inositol and palmitate ratios, it was found that there is no significant difference in the inositol content per unit membrane either within the hybrid pair 39EC13/ESH39 or between the parental lines MRC-5 and $D98AH_2$. Conversely, the value 1 is not strictly within the 95% confidence limit for the 5E/5L pair even though neither the inositol nor palmitate ratios were found to differ significantly.

However, for every cell pair, at least one experiment was performed whereby relative inositol and palmitate values were determined in parallel. Multiple replicates of both cell lines within a pair were plated and concurrently labelled with either inositol or palmitate for the 45 hours. In these cases, the ratio of membrane inositol to palmitate content of the non-tumourigenic to tumourigenic cells was always less than one. This indicates, that the assumption of the tumourigenic phenotype may, indeed, be associated with elevated levels of inositol lipids per unit membrane and that

TABLE 3.3

RELATIVE PHOSPHOINOSITIDE LEVELS PER UNIT MEMBRANE

Cell pair	Ratio of non-tumourigenic/tumourigenic total phosphoinositides per membrane palmitate	
	A	B
5E/5L	**0.80**	(0.64 , 0.99)
39EC13/ESH39	**0.83**	(0.66 , 1.06)
$Cn_2B_1Col_1$/5A7mp	**0.61**	(0.49 , 0.74)
A183/D98AH_2	**0.91**	(0.63 , 1.31)
MRC-5/D98AH_2	**0.53**	(0.41, 0.70)

Determination of the relative phosphoinositide levels per unit membrane in the non-tumourigenic and tumourigenic cells (column A) was made by dividing the mean ratio for the phosphoinositides (Table 3.1) by that for relative membrane palmitate (Table 3.2). 95% confidence intervals (column B) were obtained as stated in 3.C.5.

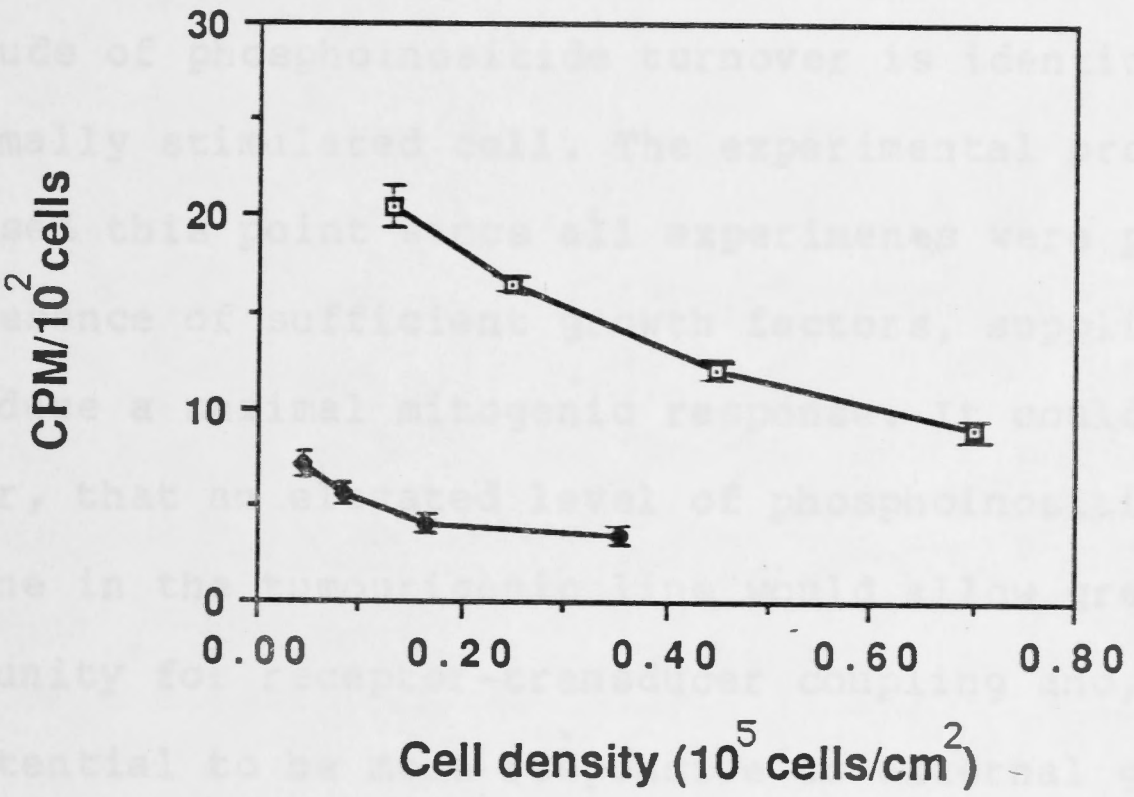
the failure to show a statistical difference is attributable to some factor which varies between experiments, generating larger deviation in absolute values than the differences between the cell types.

One factor which did differ between experiments was cell density. Although both cell lines within a pair were always assayed on the same day and plated at the same density, the total cell number per dish in experiments occurring on different days, varied slightly due to differences in plating efficiency and the time in culture before adding radio label. When the amount of radio label incorporated into 10^5 cells by 45 hours was monitored at different cell densities, it was found that both the membrane inositol and palmitate levels decreased as cell density increased (see Fig. 3.4). This phenomenon was seen for both the transformed and tumourigenic lines and did not differ between cell types. Because both inositol and palmitate values were affected, it was unlikely that the effect was related to the role of the inositol lipids in mitogenic signal transduction but was more likely due to some nutritional or spatial constraint.

It should be noted that there is no strong statistical evidence for the proposal that tumourigenesis is associated with an elevated level of inositol lipids per unit membrane. In fact, if membrane inositol content was corrected for total cellular protein instead of membrane palmitate, then the association would completely disappear. However, the idea is intriguing, particularly if it is to be postulated that a change in phosphoinositide metabolism occurs during the

FIGURE 3.4

EFFECT OF CELL DENSITY ON INOSITOL AND PALMITATE INCORPORATION INTO CELLULAR MEMBRANES



Results represent typical experiments indicating how the steady state levels of [^{3}H]-inositol (♦) and [^{14}C]-palmitate (▫), incorporated into cellular membranes varies as a function of cell density. Each point was obtained from duplicate determinations, the range of which are denoted by bars.

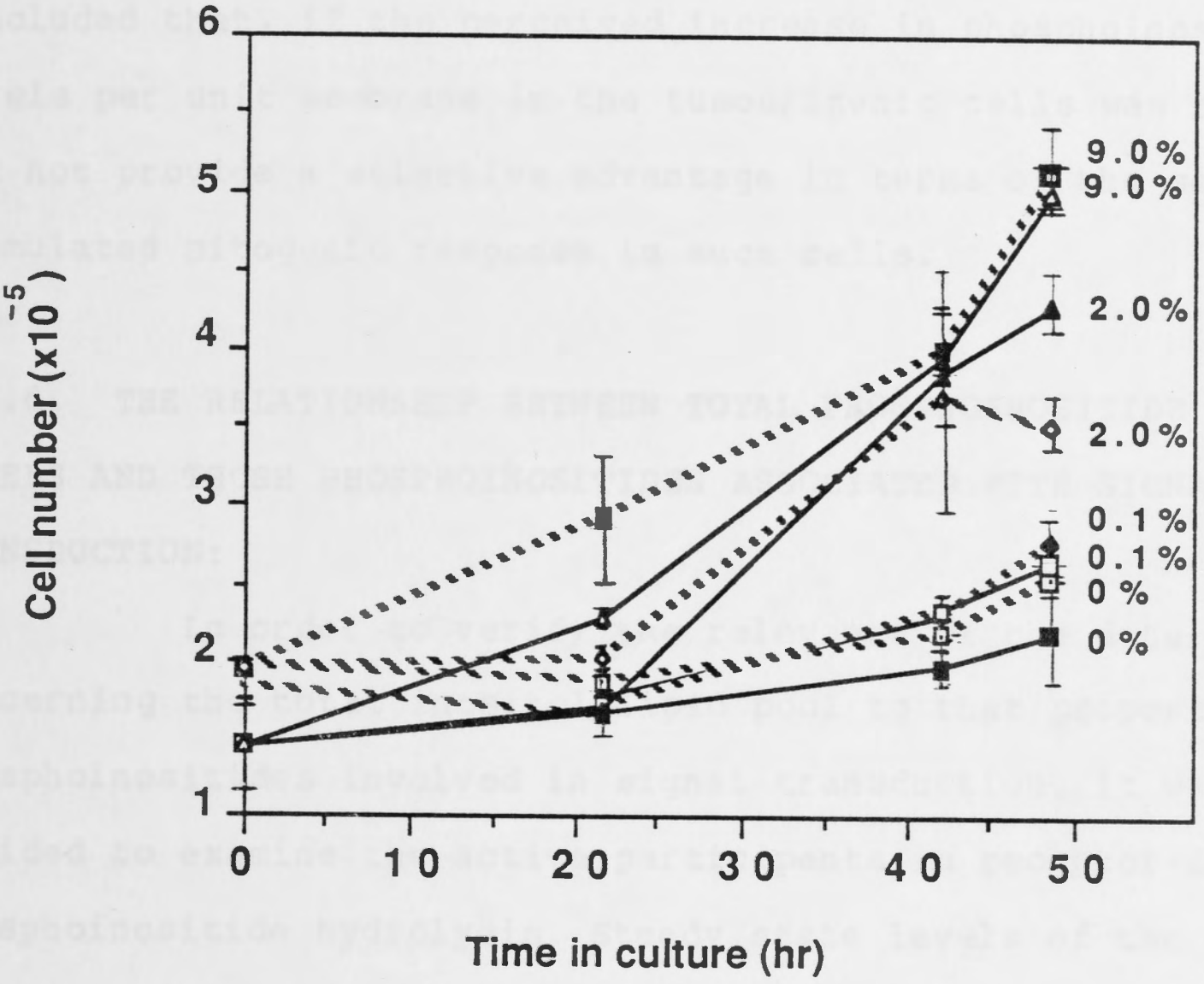
transition from the transformed to the tumourigenic, phenotype and suggests that the nature of the change at this level of growth control is more subtle than a simple overproduction of an initiating mitogenic signal.

Perhaps, instead of a continuous overproduction of second messengers, the rate of phosphoinositide metabolism in tumourigenic cells is altered as a function of differing levels of growth factor stimulation, such that, even when external growth stimuli are absent or at suboptimal levels, the magnitude of phosphoinositide turnover is identical to that of a maximally stimulated cell. The experimental protocol had not addressed this point since all experiments were performed in the presence of sufficient growth factors, supplied as serum, to produce a maximal mitogenic response. It could be proposed, however, that an elevated level of phosphoinositides per unit membrane in the tumourigenic line would allow greater opportunity for receptor-transducer coupling and, therefore, the potential to be more responsive to external growth signals. This is an important point since tumourigenicity is defined in an in vivo context where the actual level of growth stimulation from minute to minute is, as yet, unknown.

This idea was investigated by examining the growth of the cell hybrid pair 39EC13/ESH39 in medium containing different concentrations of serum (see figure 3.5). However, there was no serum concentration below which the growth of the transformed cell line was inhibited compared with its tumourigenic partner and this approach was not pursued further. Obviously such studies were preliminary and investigations

FIGURE 3.5

EFFECTS OF SERUM CONCENTRATION ON CELL GROWTH



Comparison of the rates of growth of the tumourigenic (——) and non-tumourigenic (- - - -) hybrid pair, 39EC13 / ESH39 in medium containing different concentrations of serum, as indicated. Each point represents the average of two measurements, the range of values being denoted by bars. Comparative growth studies were also performed at serum concentrations (v/v) of 0.2%, 0.5%, 0.75% and 1.3% with similar results.

involving the systematic variation of purified growth factors may demonstrate a level of external growth stimulation at which the mitogenic response of the tumourigenic cells is superior. However, the interpretation of the relevance of such findings to tumourigenic expression *in vivo* would be complex, and from the preliminary experiments performed with whole serum, it was concluded that, if the perceived increase in phosphoinositide levels per unit membrane in the tumourigenic cells was real, it did not provide a selective advantage in terms of the serum stimulated mitogenic response in such cells.

3.C.6. THE RELATIONSHIP BETWEEN TOTAL PHOSPHOINOSITIDE LEVELS AND THOSE PHOSPHOINOSITIDES ASSOCIATED WITH SIGNAL TRANSDUCTION:

In order to verify the relevance of the data concerning the total inositol lipid pool to that proportion of phosphoinositides involved in signal transduction, it was decided to examine the active participants in receptor-driven phosphoinositide hydrolysis. Steady state levels of the individual inositol lipids were determined in the hybrids after separation by thin layer chromatography. It was found that the ratio of PI to PIP_2 from 5 separate experiments was 4.2 ± 0.76 and 5.3 ± 1.3 (mean $\pm$ standard error) for the transformed and tumourigenic cell types respectively. These values were not significantly different ($P > 0.4$), indicating that the relative kinetics and pool size behaviour of the PIP_2 pool mirrors that of the total phosphoinositide pool. However, several studies in erythrocytes (Muller *et al.* (1986)) and platelets

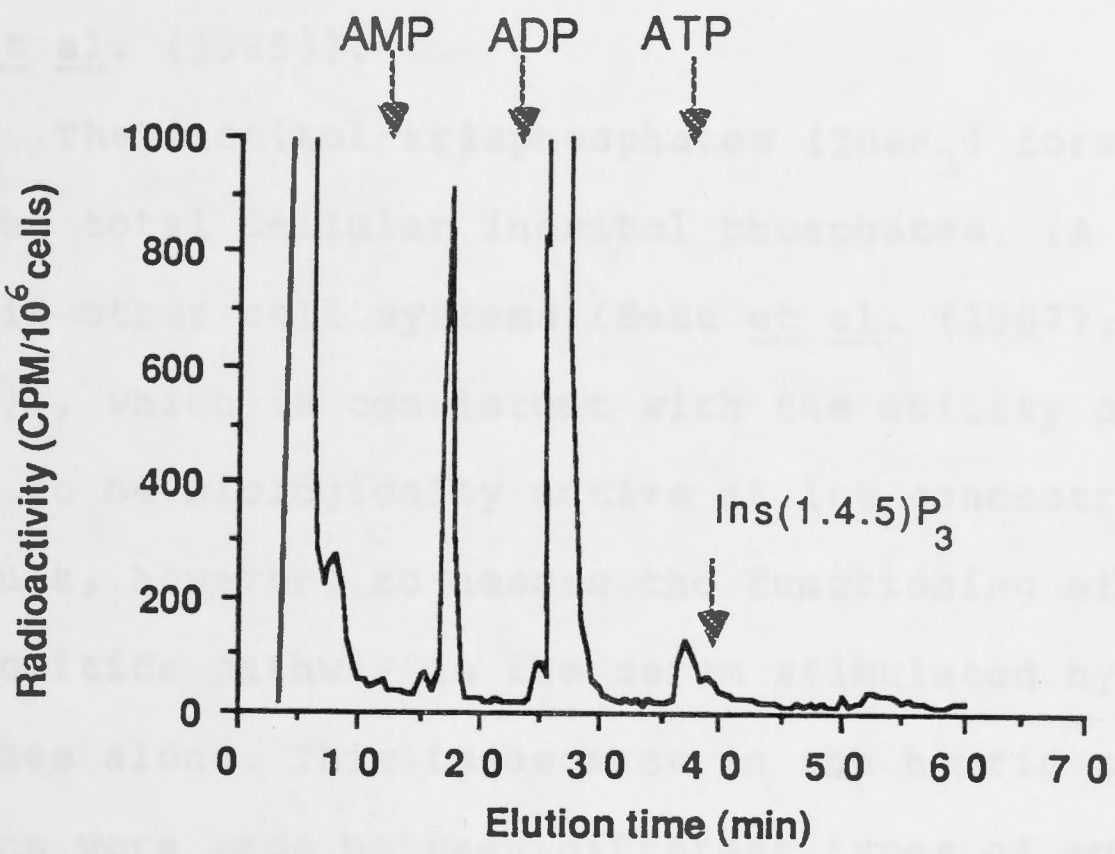
(Vickers et al. (1986)) have indicated that there are multiple metabolic pools of PIP_2, not all of which are likely to be involved in signal transduction (a finding supported by the decay experiments in the hybrids). Therefore, it was decided to complement the steady-state lipid data with an examination of the inositol phosphates.

After labelling the hybrid pairs with [^{3}H] - inositol for an extended period (72 hours) in serum supplemented medium, the cells were lysed and their aqueous-soluble inositol phosphates extracted for separation by high performance liquid chromatography (HPLC). A typical profile is given in Figure 3.6. The calcium-mobilizing inositol trisphosphate isomer, inositol(1,4,5)trisphosphate ($Ins(1,4,5)P_3$), was identified as that radioactive peak which co-chromatographed with a tritiated authentic $Ins(1,4,5)P_3$ standard. Inositol(1,3,4)trisphosphate ($Ins(1,3,4)P_3$) eluted just prior to this within half a minute of the standard adenosine triphosphate (ATP) (as reported, (Lew et al. 1986).

Although complete baseline resolution of these isomers has been reported previously (Irvine et al. (1985); Lew et al. (1986); Burgess et al. (1985)), such resolution was not achieved in the present study despite numerous modifications to the gradient system. Identification of the other peaks is tentative as authentic standards for inositol bisphosphate ($InsP_2$) and inositol monophosphate (InsP) were not chromatographed. Free inositol was chromatographed and eluted in the void volume at the position corresponding to the large initial peak. Peaks two and three though possibly

FIGURE 3.6

SEPARATION AND QUANTITATION OF THE INOSITOL PHOSPHATES



Cell line		Radioactivity/10^6 cells	
		$Ins(1,3,4)P_3$	$Ins(1,4,5)P_3$
$Cn_2B_1Col_1$	(Tr)	293	170
5A7mp	(Tu)	206	91
$Cn_2B_1Col_1$	(Tr)	81	105
5A7mp	(Tu)	73	84
5E	(Tr)	168	299
5L	(Tu)	125	299

Steady state levels of inositol phosphates in the transformed (Tr) and tumourigenic (Tu) cell hybrids were determined after separation by HPLC, as outlined in 3.B.8. A typical column profile is presented, as are results for the levels of the two isomers of inositol trisphosphate. Comparison of the levels of these isomers between the transformed and tumourigenic hybrids, using the two-sided Students t-test indicated that differences between lines were not significant.

glyceroinositol phosphates, are assumed to be $InsP_1$ and $InsP_2$ on the basis of their elution positions in relation to adenosine monophosphate (AMP) and adenosine diphosphate (ADP) (Irvine et al. (1985)).

The inositol trisphosphates ($InsP_3$) form a minor part of the total cellular inositol phosphates. (A finding also observed in other cell systems (Best et al. (1987); Hansen et al. (1986)), which is consistent with the ability of these compounds to be biologically active at low concentrations. It is difficult, however, to assess the functioning of the phosphoinositide pathway in the serum stimulated hybrids from these values alone. This is because in the hybrid study, comparisons were made between different types of equally stimulated cells, as opposed to single cell populations after different levels of stimulation. Appropriate negative controls were not readily available due to the inability of the hybrids to be growth arrested. Quiescent fibroblasts were considered an unsuitable control due to the observed size difference (affecting total phosphoinositide pools) between cells. Moreover, the presence of the inositol trisphosphate isomers does not, in itself, signify that receptor mediated phosphoinositide hydrolysis is occurring. Both $Ins(1,4,5)P_3$ and $Ins(1,3,4)P_3$ have been detected in a number of cells prior to stimulation, the levels of which substantially increase upon the addition of an agonist.

Current information concerning the agonist-stimulated production of inositol phosphates indicates several points about the metabolisms of $Ins(1,4,5)P_3$ and

$Ins(1,3,4)P_3$ which must be considered when interpreting the steady state data. The first is that $Ins(1,4,5)P_3$ is produced immediately in response to agonist stimulation but then drops, in the majority of reports, within 60 seconds to new steady state levels, even in the constant presence of an agonist (Burgess et al. (1985)). The extent of the decline varies with the cell type and the tissue examined (Irvine et al. (1985); Burgess et al. (1985): Biden et al. (1987); Hansen et al. (1986)) with levels in some cells returning almost to baseline (Irvine et al. (1985); Burgess et al. (1985)). This is possibly due, not only to the rapid metabolism of $Ins(1,4,5)P_3$ (see Majeras et al. (1988)) but also to the reported negative feedback loop involving the inhibition of phospholipase C by protein kinase C (Kikuchi et al. (1987)). Consequently, in the continuous presence of an agonist, $InsP_3$ levels are presumably cycling (see Cuthbertson and Cobbold (1985); Berridge (1987)). If this is so, the absolute levels of $InsP_3$, at a given time, are dependent on the temporal position of the measurement within the cycle., and therefore the values obtained for the population of asynchronously proliferating hybrids represent an average of the extremes of $InsP_3$ fluctuation. This would explain low levels of $InsP_3$ without the need to suggest that the phosphoinositide pathway does not function in these cells during serum-stimulated proliferation.

Secondly, some statement about the metabolism of the $InsP_3$ in the hybrids can be made by equating the relative levels of the trisphosphate isomers. From agonist stimulated

systems it appears that Ins(1,3,4)P_3 is formed from Ins(1,4,5)P_3 via Ins(1,3,4,5)P_4 (Batty et al. (1985)) (see Introduction diagram 1.3). It rises within the cell 5 seconds after agonist stimulation and in most cells remains elevated for some minutes. The relative levels of the isomers in stimulated cells varies with the time after stimulation and also with the cell type and agonist (Biden et al. (1987); Burgess et al. (1985)). However, all evidence indicates that the metabolism of Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4 is rapid, occurring within 10 seconds (Burgess et al. (1985); Biden et al. (1987)). It was noted in the hybrid study that there was a consistent delay of 90 seconds between removal of the labelling medium and TCA precipitation of the cells, a duration which is hypothetically sufficient to deplete cells of agonist generated Ins(1,4,5)P_3. This is unlikely, however, as it assumes that receptor-mediated generation of Ins(1,4,5)P_3 ceases immediately upon removal of the medium, requiring agonist-receptor dissociation to be instantaneous. Moreover, it would predict (if, as in other cell types, Ins(1,4,5)P_3 was metabolized via InsP_4) that the Ins(1,3,4)P_3 isomer would predominate over Ins(1,4,5)P_3. Instead, the hybrids displayed very similar levels of the isomers in 10 separate determinations. Alternatively, the hybrids may metabolize, Ins(1,4,5)P_3 directly to Ins P_2 and not via InsP_4. This possibility is likely since radioactivity eluting at a position consistent with InsP_4 was very low or absent in most chromatograms. In addition, the peak of radioactivity consistent with IP_2 was large. Due to its elution position in

relation to ADP it is likely to be the Ins(1,4)P_2 isomer and therefore not produced from Ins(1,3,4)P_3.

A third possibility, which again must be mentioned, is that the isomers are at comparable levels because they are equivalent to the basal levels detected in other cells, their generation being dissociated from signal transduction.

Experiments were performed to compare $InsP_3$ levels in the transformed and tumourigenic hybrids and results from three separate experiments are given in Table 3.4. It could be argued that, if anything, the levels of both isomers are slightly elevated in the transformed cells. However, these differences are minor. Moreover, values were derived from the area under the chromatograms which, as can be seen from the magnitude of the values, represent very few radioactive counts above background. More experiments will be needed in order to establish whether any noted changes lie within the range of the experimental error. However, since the $InsP_3$ levels in the different cell types are very similar, it is unlikely that the possible metabolism of the $InsP_3$ during isolation, influences the interpretation of relative $InsP_3$ levels (unless rates of metabolism are coordinately altered in the different cell types) and it is clear, from the current results, that the elevated levels of $[Ca^{2+}]_i$ reported in the tumourigenic cells (Banyard and Tellam (1985)) are not due to elevated steady state levels of Ins(1,4,5)P_3 in these cells.

3.D RAMIFICATION OF RESULTS

The results reveal that, during continuous serum stimulation, there is no difference in the metabolism of the

phosphoinositides between the transformed and tumourigenic HeLa X fibroblast human somatic cell hybrids, indicating that, as a generalized phenomenon, the maintenance of the tumourigenic phenotype does not require an alteration in phosphoinositide metabolism.

This is in contrast to a number of studies examining similar parameters to those investigated in the present study. Such studies have reported an alteration in either PIP_2:PI ratios (Fleischman et al. (1986)), steady state levels of $InsP_3$ (Jackowski et al. (1986)) or the metabolism of PI (Diringer and Friis (1977), Kubota et al. (1986)) in association with viral or chemical transformation. The most likely reason for the conflict in findings is due to the varying nature of the cell systems used.

The human somatic cell hybrid model is an internally controlled system in that tumourigenic cells can be generated from the non-tumourigenic hybrids without the additional treatment of cells with carcinogens or foreign genetic material. Their phenotype is stable and their tumourigenicity in vivo was checked regularly throughout the course of this study. Therefore, phosphoinositide metabolism was assessed in the tumourigenic hybrids and their equally transformed but non-tumourigenic partners without the additional complications generated by viral infection or non-specific chemical carcinogens. Moreover, as tumourigenesis is considered to be a multistage process, it is possible that the transformed and tumourigenic hybrids may be at different relative sub-stages in the progression to the tumourigenic process from the cells in

the viral and chemical transformation studies. It may be that an alteration in the phosphoinositide pathway did occur at an initial stage in the transition from normal to the fully tumourigenic phenotype in the HeLa parent but, if this was the case, the present study suggests that the maintenance of the altered phenotype does not require continuous repetition of the initiating event.

It should also be noted, however, that there is very little difference in the _in vitro_ growth characteristics in terms of growth-factor requirements and doubling times between the hybrids (Stanbridge _et al_. (1982), figure 3.4). Although there are clear distinctions between the transformed and tumourigenic hybrids _in vitro_ (including alterations in the cytoskeleton (Der _et al_. (1981), Gowing _et al_. (1984)), glucose transport (White _et al_. (1983)) and intracellular free calcium (Banyard and Tellam (1985)) which are presumed to relate to differences in tumourigenic expression, it may be that the effects of the proposed tumour suppressor genes (Stanbridge (1985)) upon cell growth are not fully expressed under _in vitro_ conditions and that differences in the phosphoinositide pathways between the two cell types would be manifested in the whole animal where a greater variety of external stimuli may arrive at the cell receptors. It is interesting, therefore, that the only consistent difference in phosphoinositide metabolism within the hybrid pairs was that the levels of phosphinosites per unit membrane were elevated in the tumourigenic lines, suggesting that they may have greater potential for signal transduction under certain growth stimulation conditions.

Another possibility is that the phosphoinositide pathway in the hybrid cells, is altered at a level beyond the inital signal transduction event. If the maintenance of the tumourigenic state in all cell systems requires a change in a specific cellular pathway, then, since tumourigenesis in the somatic cell hybrids is associated with the loss of genetic material rather than the gain of some foreign gene product (which may act directly on the pathway), the hybrid cells would have to alter the expression of their own proteins to mimic that change. From the examination of phosphoinositide metabolism in the hybrids, it appears that the enzymes associated with the metabolism of PIP_2 and $InsP_3$ are not altered. However, intracellular calcium levels have been reported to be altered in the cells (Banyard and Tellum (1985)), an effect which may be due to an alteration in membrane-located ion channels. It is possibly significant in this context that a number of proteins associated with the movement of Ca^{2+} across membranes can be altered by the activation of protein kinase C (Lagast et al. (1984); Strong et al. (1987)), an enzyme which is activated by a metabolite of phosphoinositide breakdown.

PKC has been proposed to have a general regulatory role in a number of cell processes (Berridge (1987)), including both proliferation and differentiation. As it has been proposed that the tumourigenic hybrids do not stop growing in vivo because they fail to differentiate (Stanbridge et al. (1983); Harris (1985)), it is logical to examine several aspects of the enzyme, protein kinase C, in the somatic cell hybrids. This work is described in the next chapter.

CHAPTER 4:

PROTEIN KINASE C CONTENT, ACTIVITY AND SUBCELLULAR DISTRIBUTION IN THE TRANSFORMED AND TUMOURIGENIC CELL HYBRIDS: Investigation of Possible De-regulation at the Level of the third Messenger

4.A INTRODUCTION

There is no clear association between the assumption of the tumourigenic phenotype and an alteration in phosphoinositide metabolism in the human somatic cell hybrids (see Chapter 3). This does not, however, discount the possibility that the phosphoinositide pathway may be disrupted in the tumourigenic cells at a level beyond that of the initial signal transduction event.

One of the most intriguing features of the phosphoinositide pathway is that two separate second messengers are produced from the one signal transduction event (Berridge (1987)). The rise in intracellular free calcium ($[Ca^{2+}]_i$) generated by inositol(1,4,5)trisphosphate and the activation of protein kinase C (PKC) by 1,2-sn diacylglycerol (DAG) have been shown not only to modulate one another but also to act synergisticly to generate the growth response (see Introduction 1.C.2.5). However, it is also apparent that the two functional arms of the pathway can operate independently of each other to influence cellular growth. Just as agents, such as glucagon which stimulate cyclic adenosine 3'5'-monophosphate (c-AMP) production, but not phosphoinositide hydrolysis, can raise

$[Ca^{2+}]_i$ (Poggioli et al. (1986)), so, too, can DAG levels be elevated without an accompanying change in phosphoinositide metabolism. Both insulin and the tumour-promoting phorbol ester, phorbol 12-myristate 13-acetate (PMA) have been shown to increase DAG levels in myocytes (Farese et al. (1985)) and fibroblasts (Takuwa et al. (1987)) respectively, without an increase in inositol phosphates. This is most likely achieved by an increased turnover of other phospholipids such as phosphatidylcholine and inhibition of DAG kinase (Takuwa et al. (1987)). Given the reported elevation in $[Ca^{2+}]_i$ in the tumourigenic hybrids, it is conceivable that, if the activity of PKC in these cells was also modified, then such cells could generate their own aberrant growth signals without the need for a disruption in phosphoinositide metabolism.

Protein kinase C, the effector molecule for the second messenger, DAG, is a Ca^{2+}- and phospholipid-dependent protein kinase which, when activated, has been shown to mediate a wide variety of cellular responses (Kikkawa and Nishizuka (1986)). There are several logical reasons to predict a role for the alteration in the activity of this enzyme at some stage in the tumourigenic process.

Firstly, there is the association between the mode of action of a number of structurally unrelated tumour promoters and PKC (Kikkawa and Nishizuka (1986); Nishizuka (1984)). Tumour promoters are molecules that are not carcinogenic themselves although they are able to increase the incidence of tumours in animals pre-treated with carcinogenic agents. This suggests that they act by promoting the transition

from a de-stabilized (initiated or transformed) to a fully tumourigenic growth state. Some of the most effective tumour promoters are the tumour promoting phorbol esters, such as PMA which are thought to act predominantly through their ability to bind to and modulate the activity of PKC (Nishizuka (1984)).

Secondly, an alteration in membrane fluidity and lipid composition is one of the most common features of tumourigenic cells (e.g. Itaya et al. (1976)). Given the phospholipid dependency of PKC (Kaibuchi et al. (1981)), this membrane disruption may serve to modify enzyme activity even in the absence of altered DAG levels. Moreover, the elevated $[Ca^{2+}]_i$ in the tumourigenic hybrids (Banyard and Tellam (1985)) is likely, in itself, to alter the activity of many cellular enzymes, including PKC. It is difficult, however, to assess the extent to which this elevation in $[Ca^{2+}]_i$ would affect PKC, given that the calcium measurements represent average cytoplasmic calcium concentrations for a population of cells. Nevertheless, even small changes in calcium concentration can affect PKC regulation, since translocation to the membrane increases most sharply between 100nM and 500nM free calcium (Wolf et al. (1985)).

Further support for the concept that a change in PKC activity occurs in association with the expression of tumourigenicity can be drawn from the correlation of reported differences between the transformed and tumourigenic hybrids with known actions of PKC. A number of phenotypic differences between the transformed and tumourigenic hybrids have been noted, including changes in cytoskeletal organization (Der et

al. (1981); Gowing et al. (1984)), fibronectin and collagen cell surface distribution (Stanbridge et al. (1982)), glucose transport (White et al. (1983)), hormone secretion (Stanbridge et al. (1982)) and $[Ca^{2+}]_i$ (Banyard and Tellam (1985)).

It is noteworthy that PKC phosphorylates several of the cytoskeletal binding proteins, including vinculin (Werth et al. (1983)) and talin (Litchfield et al. (1986)), which are responsible for organization of the cytoskeleton within the cell. Moreover, treatment of cells with PMA results in cytoskeletal re-organization similar to that generated (transiently) by the mitogen, platelet derived growth factor (PDGF) (Herman and Pledger (1985); Herman et al. (1986)) and to that displayed by the tumourigenic cells (Gowing et al. (1984)). In addition, given the co-ordinate alteration in the tumourigenic cells of the two major extracellular matrix components, fibronectin and collagen (Stanbridge et al. (1982)), it is pertinent that long-term treatment (> 6 hr.) of human fibroblasts with PMA increases the secretion of basement membrane (type IV) collagen-degrading metalloproteinase (Salo et al. (1985)). PKC has also been shown to phosphorylate the glucose transporter both *in vivo* and *in vitro* (Witters et al. (1985)) and regulates the expression of a number of genes including c-*fos*, the expression of which is elevated in the tumourigenic $D98AH_2$ parent (O'Hara et al. (1986)).

Due to the key role played by PKC in co-ordinating the growth response in normal cells (Berridge (1987; 1987a)) coupled with the circumstantial evidence that a PKC-like activity may be de-regulated in the tumourigenic lines, it was

decided to investigate several aspects of this enzyme in the somatic cell hybrid system. Because mitogenic stimulation and cellular growth state (quiescence versus logarithmic growth) have been associated, in a number of systems, with the translocation of cytosolic PKC to the membrane (see Section 1.C.2.4.6), it was decided to monitor levels of PKC activity in cytosolic and membrane fractions from the hybrid cells and the parental lines. In addition, phorbol diester binding studies were performed with intact cells, as an independent measure of the molecular levels of PKC in the different cell types.

4.B MATERIALS AND METHODS

4.B.1 ISOLATION OF PROTEIN KINASE C:

Cell pairs were grown to confluency in large 175cm^2 tissue culture flasks in appropriately supplemented DMEM (see Chapter 2: General Methods). At the time of isolation, the medium was removed and the cells washed with 2x100mL of ice-cold normal saline. Cells were then scraped into 10mL of ice-cold extraction buffer containing:

20mM Tris/HCl pH 7.4-7.5

0.25M Sucrose

1mM dithiothreitol (DTT)

10μg/mL leupeptin

100μM phenylmethylsulfonyl fluoride (PMSF)

(DTT, leupeptin and PMSF were stored at -20°C as stock solutions: 1M DTT in H_2O, 10mg/mL leupeptin in H_2O and 10mM PMSF in ethanol, and added just prior to use.)

A small sample of the collected cells was taken for determination of cell number and the remainder was sonicated on ice for 2x10 seconds, on setting 5 of a sonicator (Ultrasonics Inc.; Model W-220F). A known volume of this cell homogenate was then centrifuged at 4°C for 1 hour at 100 000g in a 50Ti rotor using a Beckman ultracentrifuge (model L3-50 or L8-70). The resultant supernatant was considered to be the cytosolic fraction. The pellet was resuspended in a buffer containing:

20mM Tris/HCl pH 7.4-7.5

0.25M Sucrose

0.5mM EDTA

0.5mM EGTA

0.05% (v/v) triton X100

1mM DDT

10μg/mL leupeptin

100μM PMSF

and tumbled at 4°C for 45 minutes before being centrifuged for 1 hour, 4°C, at 100000g. The supernatant from this centrifugation was considered to contain the membrane bound PKC (referred to as the membrane fraction).

Protein kinase C was partially purified from cytosolic and membrane fractions by DEAE anion exchange column chromatography performed at 4°C in a manner similar to that outlined previously (Miloszewska et al. (1986)). Glass columns 20cm x 1cm diameter (from Bio.Rad, USA) were packed with approximately 10mL of DE 52 cellulose (from Whatman Ltd., U.K.) and pre-equilibrated with at least 400mL of column buffer comprising 20mM Tris/HCl pH 7.4-7.5, 0.5mM

ethylene-diaminetetraacetic acid (EDTA), 0.5mM ethylene glycol bis (β-aminoethylether) - N,N,N',N'- tetraacetic acid (EGTA). Pre-equilibration was found to be essential for the successful elution of PKC. Immediately before loading the sample, 15mL of column buffer containing freshly added 1mM DTT, 10μg/mL leupeptin and 100μM PMSF was passed through the column and, after the sample had been loaded, another 15mL of the same buffer was used to wash the column free of unbound protein. Confirmation that all unbound protein had been eluted was obtained by monitoring elution absorbance at 280nm. Protein kinase C was then eluted with a 40mL continuous gradient of 0-0.4M NaCl in column buffer. Again, protease inhibitors and protein stabilizers (10μg/mL leupeptin, 50μM PMSF and 1mM DTT) were included in this step. Fractions were collected every 3 minutes ($\cong$ 2mL/fraction) and assayed for PKC activity as outlined below. At all stages during the procedure, cellular preparations were maintained at 4°C.

4.B.2 DETERMINATION OF PROTEIN KINASE C ACTIVITY:

PKC activity in the column fractions and the samples from which they were derived was assayed in a manner similar to those outlined previously (House et al. (1987)). The substrate used, termed GS(1-12) was developed and generously provided by Dr. Kemp (Department of Medicine, The University of Melbourne, Victoria, Australia). GS (1-12) is a 12 amino acid peptide analogue of glycogen synthase (sequence Pro-Leu-Ser-Arg-Thre-Leu-Ser-Val-Ala-Ala-Lys-Lys). The advantage of using such an enzyme-specific, high affinity substrate (House et al (1987)),

is that the phosphorylating activity monitored in the absence of phosphatidylserine is likely to be attributable to protein kinase M; (PKM is that kinase produced after the phospholipid and calcium binding regulatory domain of PKC has been removed (Inoue et al (1977)).

PKC activity was assessed by determining the amount of ^{32}P incorporated into the substrate, GS(1-12), over a 15 minutes period in the presence or absence of phosphatidylserine (PS). All column fractions were assayed within 24 hours of separation. The [γ-^{32}P]-ATP used as the phosphate donor (New England Nuclear, USA through DuPont, USA) was supplied at high specific activity (111 TBq/mmol; 3000 Ci/mmol) but was diluted before use in 10μM ATP in 10mM Tris/HCl, pH 7.4 to give $\cong$ 250 000 CPM/reaction tube. (All ATP was stored at -20°C). PS was stored in chloroform: methanol (95:5 (v:v)) at -20°C. 500μg lots were dried down under nitrogen before use and re-solubilized in 20μL of chloroform: methanol (1:1 (v:v)). One mL of 10mM Tris/HCl pH 7.4 was added and the solution sonicated 2x10 seconds on setting 5 of the sonicator to produce a milky micellar solution. The substrate was solubilized in H_2O. A 10 x stock of reaction buffer containing 100mM Tris/HCl pH 7.4, 100mM $MgCl_2$, 7.5mM $CaCl_2$ was stored at room temperature.

Assays were carried out in polycarbonate plastic tubes in a final volume of 70μL.

The reaction mixture containing:

	FINAL CONCENTRATION
7μL 10 x reaction buffer	0.75mM $CaCl_2$, 10mM $MgCl_2$ 12mM Tris/HCl*
$\pm$ 7μL 500 g/mL PS	50μg/mL
10 or 17μL H_2O	-
5μL 1mg/mL substrate	71μg/mL
7μL 100μM [^{32}P]-ATP	10μM

(* after addition of column fractions, final conc. 22mM Tris, 0.5mM $CaCl_2$, 9.75mM $MgCl_2$)

was pre-warmed at 30°C for 10 minutes before addition of 34μL of sample. The solution was then vortexed and incubated for 15 minutes at 30°C. The reaction was stopped by spotting 55μL of reaction mixture onto a 2.5cm x 2.5cm square of P81 cellulose phosphate paper (Whatman Ltd., U.K.), allowing approximately 10 seconds for the solution to soak into the paper and then dropping the paper into a wire mesh basket inserted into a beaker containing 75mM orthophosphoric acid. When all fractions from a column run had been processed, the papers were soaked for a further 10 min in the orthophosphorate, washed for 5 minutes in fresh acid and then rinsed for 2 minutes in absolute ethanol before being dried under a hair dryer. The papers were then counted for radiolabelled peptide after addition of scintillation fluid (67% (v/v) xylene, 33% (v/v) triton X114, 0.5% (w/v PPO) by standard liquid scintillation counting.

4.B.3 PHORBOL ESTER BINDING STUDIES:

Phorbol ester binding was performed using [20(n)-^{3}H]-Phorbol-12,13-dibutyrate, specific activity 729 GBq/mmol (19.7Ci/mmol) (from Amersham Int. Aust.). Non-specific binding was judged as that level of binding occurring in the presence of 5μg/mL (9.9μM) unlabelled phorbol-12,13-dibutyrate (PDBu) (Sigma Chemical Co., USA).

Cell pairs were plated at either 0.5×10^5 or 1.0×10^5 cells per dish in 35mm diameter plastic petri dishes and grown for 24-48 hours in appropriately supplemented DMEM. At the time of labelling, the medium was removed and 0.5mL of the same medium, supplemented with an appropriate concentration of [^{3}H]-PDBu, with or without unlabelled PDBu., was added. The dishes were then returned to the 37°C incubator for 30 minutes.

After this time, the medium was rapidly removed and the dishes washed with 3X 1mL of ice-cold phosphate buffered saline (PBS) (see Appendix for formula). The dishes were then stored on ice prior to trypsinization (as in Chapter 2: General Methods). 4 millilitres of the 0.5mL cell suspension was transferred to liquid scintillation vials for determination of radioactivity. Cell numbers were determined from parallel dishes.

For Scatchard analysis, binding was determined at 0, 2, 5, 10, 20, 35, 50, 75 and 100nM [^{3}H]-PDBu. Single point determinations were made in quadruplicate at 50nM [^{3}H]-PDBu.

Specific binding was calculated from the formula

$$\text{B specific} = \text{B without cold PDBu} - \left[\text{B with cold PDBu} \times \frac{\text{F without PDBu}}{\text{F with PDBu}}\right]$$

where B = bound (CPM) and F = free (CPM).

The implicit assumption of this formula, that non-specific binding is a linear function of concentration, was confirmed during the course of the study, see figure 4.4.

4.C RESULTS AND DISCUSSION

4.C.1 PROTEIN KINASE C ACTIVITY IN THE CYTOSOL AND MEMBRANE OF TUMOURIGENIC AND NON-TUMOURIGENIC CELLS:

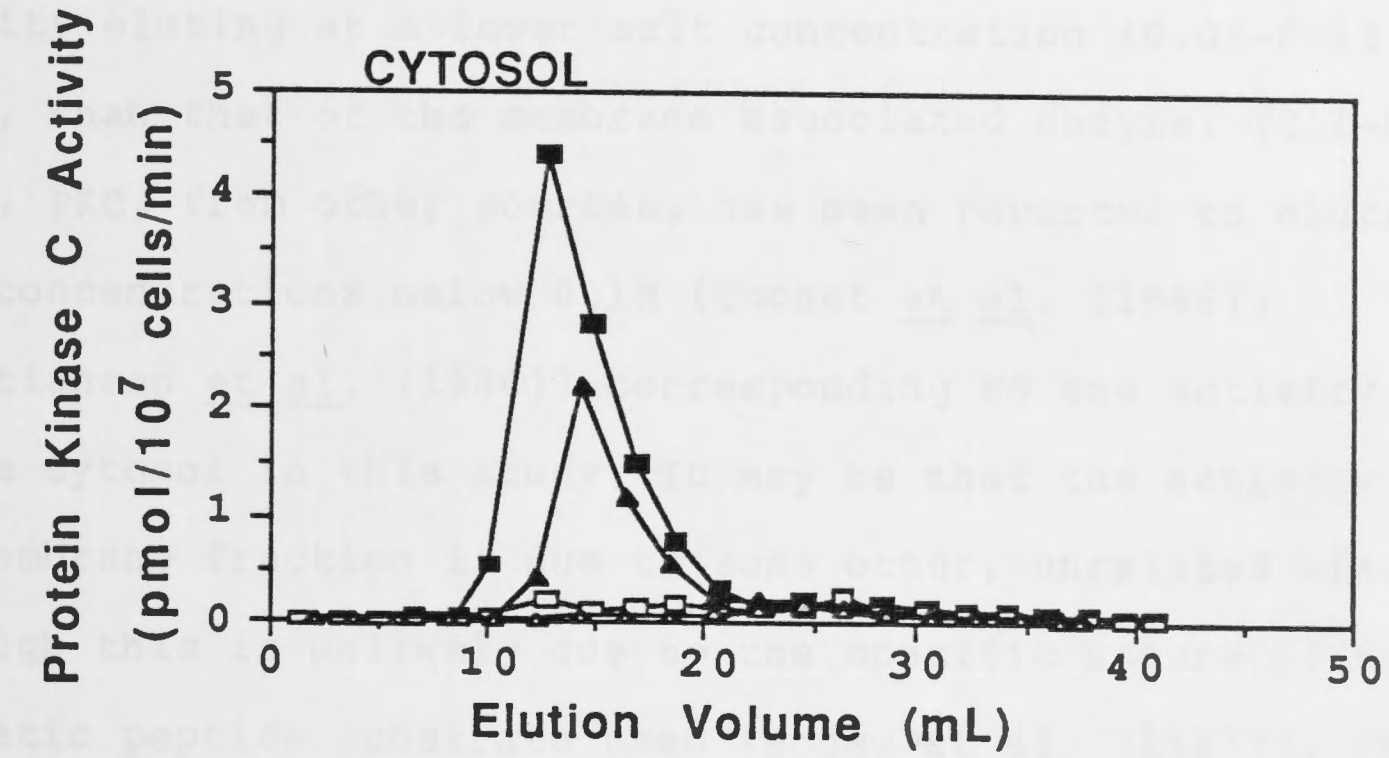
4.C.1.1 PKC Activity Profiles Generated from DE 52 Chromatography of Cytosolic and Membrane Fractions

Cytosolic and membrane-bound PKC activities (defined as that kinase activity capable of phosphorylating a specific peptide substrate in a phosphatidylserine (PS) dependent manner) were determined in column fractions from salt eluates of DE 52. Several pairs of cells were examined and typical activity profiles, for a somatic cell hybrid pair (39ECl3/ESH39), are given in figure 4.1. From these profiles several conclusions can be drawn.

The first, is that there is no difference in either the cytoplasmic or membrane activity profiles between the tumourigenic and non-tumourigenic cell types, suggesting that, if the tumourigenic lines do contain an aberrant form of PKC, the alteration does not influence its behaviour on anion exchange chromatography. This does not, however, exclude the possibility that the different cell types express different levels of the transcriptionally distinct forms of PKC [α, βI, βII and γ (Coussens et al. (1986)] since the different isozymes of C-kinase are not distinguished by DE 52 chromatography (Huang et al. (1986)).

FIGURE 4.1

PROTEIN KINASE C ACTIVITY PROFILES FOR THE MEMBRANE AND CYTOSOLIC FRACTIONS FROM A CELL HYBRID PAIR



Protein kinase C activity profiles for cytosolic and membrane extracts from the cell hybrid pair 39EC13 / ESH39 obtained after fractionation on DE 52 ion exchange resin, as outlined in methods section 4.B Enzyme activity was assessed in the presence (39EC13 ■ ;ESH39 ▲) or absence (39EC13 □ ;ESH39 △) of phosphatidylserine . Note scale difference between ordinates of cytosol and membrane profiles.

A second point is that there is a difference in the chromatographic behaviour of PKC activity between the membrane and cytosol of all cell types, with the peak cytosolic enzyme activity eluting at a lower salt concentration (0.05-0.12 M NaCl), than that of the membrane associated enzyme, (0.1-0.2 M NaCl). PKC, from other sources, has been reported to elute at salt concentrations below 0.1M (Cochet et al. (1986); Christiansen et al. (1986)) corresponding to the activity found in the cytosol in this study. It may be that the activity in the membrane fraction is due to some other, unrelated kinase, although this is unlikely due to the specific nature of the synthetic peptide substrate used (House et al. (1987)). It was noted, however, that a substantial proportion of the latter half of the membrane activity peak is PS independent, suggesting that the PKC-like activity may be attributable to the degraded form of the enzyme, known as protein kinase M (PKM) (Inoue et al. (1971); Kishimoto et al. (1983)). This kinase is produced by the proteolytic cleavage of the PS and Ca^{2+} binding regulatory domain from PKC and has, in fact, been reported to elute at a higher salt concentration than PKC (Tapley and Murray (1985); Melloni et al. (1986); Buday et al. (1987); Romhanyi et al. (1985)), further supporting the notion that some of the membraneous activity is that of PKM. There are, also, two PS dependent peaks of PKC activity in the membrane fraction. The first minor peak co-elutes with the cytosolic peak and may be due to cytosolic contamination of the membrane pellet. The fact that the second peak begins eluting at slightly higher salt concentrations than the cytosolic

enzyme is most likely due to its interaction with some solubilized membrane components, leading to either a conformational change in the enzyme or the binding of additional charged groups. However, the possibility that the PKC which associates with the membrane, is distinct from the majority of that in the cytosolic pool, cannot be excluded.

The third point concerns the observation that, in terms of proportions of activity in the subcellular fractions, the membrane fraction contained a much higher proportion of its total enzyme activity as the alleged PKM. This is surprising, since it has been reported (Melloni et al. (1986), Mizuta et al. (1985)) and it is logical to assume, given the presence of cytoplasmic substrates for PKC activity (White et al. (1984)), that cleavage of PKC at the membrane would result in the release of the catalytic domain into the cytosol, presumably leaving the calcium and phospholipid binding domain in the plane of the membrane. However, PKM activity has also been reported in the membrane fraction by others (Tapley and Murray (1985); Buday et al. (1987)). The fact that it remains associated with the membrane may indicate that, despite the presence of protease inhibitors, the PKM was generated after isolation and that the cytosol failed to show similar proportions of PKM due, simply, to the predominantly membraneous location of the appropriate cleavage enzyme (Melloni et al. (1986), Buday et al. (1987)). An alternative, less likely, explanation is that the catalytic domain forms a sufficiently strong association with the membrane to remain membrane-bound during isolation, suggesting that the catalytic domain also contains a membrane binding site.

Finally, the non-tumourigenic and tumourigenic cell types have differing amounts of PKC activity in both cytosolic and membrane extracts. Originally, it was planned to make multiple activity determinations on the un-chromatographed subcellular fractions but this was not possible as the cytosolic fractions exhibited no apparent PKC activity prior to fractionation on DE 52 cellulose, a finding discussed in more detail in section 4.C.2. Instead, the amounts of PKC and PKM in the subcellular extracts were determined by measuring the areas under the peaks of the elution profiles. These are presented in Table 4.1A and 4.1B.

4.C.1.2 Quantitation of Protein Kinase C and Protein Kinase M Activity in the Membrane and Cytosol of the Tumourigenic and Non-Tumourigenic Lines

Table 4.1A lists absolute activity values for each cell line from individual experiments. The magnitude of these activities was in agreement with those reported previously for human and mouse fibroblasts (Adamo et al. (1986)), and the trends in subcellular distribution and relative amounts of enzyme activity in the different cell types (given in Table 4.1B) were, with one exception, maintained between experiments. The exception concerned the subcellular distribution of PKC in a single experiment with 5E cells. Otherwise, the amount of PKC activity, in each cell line, was substantially higher in the cytosol than in the membrane. In the case of the single 5E experiment, cytoplasmic levels were comparatively reduced whereas membrane levels were significently higher than in other

TABLE 4.1 A

PROTEIN KINASE C ACTIVITY IN THE MEMBRANE AND CYTOSOL

Cell type	Protein kinase C activity (pmol/min/10^6 cells)			
	Cytosol		membrane	
	PKC	PKM	PKC	PKM
5E	32.8	1.8	6.0	4.2
	22.6	3.7	43.9	7.4
5L	17.0	1.4	4.7	3.3
	nd	nd	13.4	5.0
39EC13	55.0	5.4	13.9	9.8
	nd	nd	8.5	7.6
ESH39	27.5	3.4	3.9	1.6
	nd	nd	1.6	0.9
$Cn_2B_1Col_1$	9.9	1.2	5.0	2.7
	26.1	3.0	nd	nd
5A7mp	32.8	4.9	1.9	1.6
	42.9	3.5	nd	nd
MRC-5	36.1	-	nd	nd
	95.5	8.0	18.3	4.5
$D98AH_2$	12.2	1.5	nd	nd
	12.2	2.0	2.4	1.7

Protein kinase C(PKC) and protein kinase M (PKM) activity values for the membrane and cytosolic fractions of the different cell types were determined by summating the activity found in individual column fractions obtained from DE 52 anion exchange chromatography ,as outlined in 4.B.1. PKC activity was calculated as that level of phosphatidylserine(PS)-dependent kinase activity above that observed in the absence of PS. PKM was calculated as that PS-independent activity occuring above background. Non-tumourigenic and tumourigenic cells within a pair were always assayed together (nd=activity not determined)

TABLE 4.1 B

RELATIVE PROTEIN KINASE C ACTIVITY IN NON-TUMOURIGENIC AND TUMOURIGENIC CELLS.

Cell type	Relative protein kinase C activity		
		Non-tumourigenic/tumourigenic	
	cytosol/membrane	cytosol	membrane
5E	1.0		
		1.9	2.3
5L	2.3		
39EC13	2.5		
		2.0	5.0
ESH39	5.6		
$Cn_2B_1Col_1$	1.4		
		0.5	2.2
5A7mp	10.8		
MRC-5	4.5		
		5.4	5.6
$D98AH_2$	3.4		

Values represent relative enzyme activities after pooling protein kinase C (PKC) and protein kinase M (PKM) activities. A comparison between membrane and cytosolic pools was only made from those values obtained within the one experiment. Relative non-tumourigenic and tumourigenic values were derived from all relevant data.

cells studied. This may indicate that a translocation of enzyme from the cytosol to the membrane had taken place, suggesting that the membrane and cytosolic pools are freely interchangeable. However, it is possible that this result is anomalous due to an experimental artifact, such as resealing of membrane fragments after sonication, resulting in the entrapment of cytoplasmic activities within membrane vesicles. Alternatively, as the extraction buffer did not contain chelators, the presence of contaminating calcium may have resulted in the association of PKC with the membrane.

The observation that, in the majority of cases, the size of the cytosolic pool exceeded that of the membrane-associated pool was surprising, considering the number of reports indicating that proliferating cells contained relatively higher levels of C-kinase in their particulate fractions (Adamo et al. (1986); Averdunk and Günther (1986); Farrar and Anderson (1985)). A report by Adamo et al. (1986), examining a number of cell lines, indicated that, in proliferating cells, 60-80% of activity was found associated with the membrane, a situation which was reversed in confluent quiescent cultures. Although the hybrid cells in the present study were grown to high density, all cell lines were considered to be actively growing at the time of assay. One notable difference between this and the Adamo study, however, was that, in the latter case, subcellular fractions were not further fractionated prior to assay which may have influenced the interpretation of cytosolic PKC levels (see next section). An alternative report by Donnelly et al. (1985) examining PKC

distribution in fibroblasts grown in normal or low calcium medium, indicated, in agreement with the hybrid study, that after fractionation on DE 52 cellulose, cells growing in normal medium contained 90% of their PKC activity within the cytosol. Lowering extracellular calcium acted to growth arrest the cells and reduce the levels of membrane associated PKC even further. One drawback of that study, however, was that cells were disrupted in the presence of EGTA which may also have influenced relative cytosolic PKC levels (see next section).

Another consistent trend, discussed earlier, was that although the amount of PKM in the cytosolic and membrane fractions was similar, the proportion of PKM activity was, in general, 3 to 4 fold higher in the membrane fraction. As this may have been due to "post- isolational" degradation, PKC and PKM values were combined before relative values for subcellular distribution and pool sizes for the different cell types were determined.

4.C.1.3 Relative Protein Kinase C Activities in the Transformed and Tumourigenic Hybrids and Parental Lines

When activity levels in the different cell types were compared, an important trend emerged. It was found that the levels of membrane-associated PKC activity were consistently less in the tumourigenic cells compared with their non-tumourigenic partners. This is particularly important since, as PKC activity under in vivo conditions is dependent on both DAG and phospholipid, (Kishimoto et al. (1980), it is expected that it is the membrane-associated enzyme which

mediates the biological response. The fact that cytoplasmic PKC activity levels were also decreased in 3 tumourigenic cell lines, suggests that the observed decrease in membrane levels in the tumourigenic cells is due to a decrease in total cellular PKC activity, not just a change in the levels of one or more regulatory factors responsible for mediating the translocation of the enzyme to the membrane. However, since the relative total cellular PKC activities are lower than the relative membrane activities, the tumourigenic cells may also have alterations in the regulatory mechanisms associated with the translocation of the enzyme to the membrane. Intracellular free Ca^{2+} is an unlikely candidate as $[Ca^{2+}]_i$ is reported to be elevated in the tumourigenic hybrids. Therefore, DAG levels may be decreased in the tumourigenic cells.

Alternatively, however, it may be that the tumourigenic hybrids have reduced levels of one of the isozymes of PKC which preferentially associates with the membrane, thereby explaining why relative membrane levels do not reflect total cellular levels.

There are several possible reasons for this observed decrease in total enzyme activity in the tumourigenic cells. It may be due to a decreased rate of synthesis or an increase in the degradation of the enzyme. Since degradation is thought to occur primarily at the membrane (Melloni et al. (1985) this would be another explanation for the disparity between relative total and membrane-associated activities. Alternatively, the different cell types may have comparable levels of the enzyme but the rate at which the PKC molecules from the tumourigenic

cells phosphorylate the synthetic substrate may be reduced, due, perhaps, to a change in the primary sequence of the enzyme. A third possibility is that the tumourigenic lines have higher levels of phosphatase activity rather than lower levels of C-kinase activity. This is an unlikely explanation, however, since relative PKC activities were assessed after fractionation on DE 52. In order for phosphatase levels to influence the interpretation of kinase activities, they would have to co-elute with PKC. In addition, since in three cell lines the cytosolic PKC levels were also reduced, it would have to be predicted that, either the one phosphatase co-distributes between cytosolic and membrane locations, or that more than one phosphatase was elevated.

In summary, using salt gradient elution from DE 52 anion exchange resin, there was no apparent difference in the chromatographic behaviour of PKC and PKM activities between the normal, transformed and tumourigenic cell lines. However, the tumourigenic cells had consistently less membrane-associated PKC activity than their nontumourigenic counterparts. Since this difference was observed in every hybrid pair examined, it is inferred that a decrease in PKC activity is associated with the transition from the transformed to the tumourigenic phenotype. Given the well documented involvement of PKC in mitogenesis (see Berridge (1987); Kikawa and Nishisuka (1986)) and the tumour promoting actions of the phorbol esters, coupled with several of the phenotypic changes associated with the assumption of the tumourigenic state, it was predicted that any

change in PKC activity associated with tumourigenesis would involve an increase rather than decrease in PKC activity. However, PKC has also been documented to have a role in the inhibition or regulation, as well as the generation, of cell proliferation. Its function in this capacity will be discussed in conjunction with the findings from the phorbol ester binding data in section 4.C.4.

4.C.2 THE EFFECTS OF DIVALENT METAL CHELATORS ON THE SUBCELLULAR DISTRIBUTION OF PROTEIN KINASE C:

Initially, when the system was being developed, cells were isolated and disrupted in a Tris buffer containing the chelators, EDTA and EGTA (0.5mM of each), on the premise that the majority of investigators, including several examining the subcellular distribution of the enzyme, added such chelators (usually 2-5mM) to isolation buffers (Christiansen et al. (1986)). However, when cellular extracts were assayed prior to separation by DE 52 chromatography, it was routinely found, in contrast to the situation in the absence of chelators, that although activity was detected in the cell homogenate and cytosolic fraction, no apparent PKC activity was present in the membrane extracts. This was surprising considering the reported association of PKC with the membrane in actively dividing cells (Adamo et al. (1986); Farrar and Anderson (1985); Farrar et al. (1985)). Therefore, the possibility that the absence of membrane associated C-kinase activity was an experimental artifact, was investigated. The majority of activity in the cell homogenate was found in the cytosol, indicating that the

absence of membrane activity was more likely to be due to displacement, rather than inhibition or inactivation of the enzyme. This was difficult to fully assess quantitatively, however, particularly if the membraneous activity was small, because the homogenate activity was independent of exogenously added PS and the different cellular fractions contained different levels of endogenous substrate.

A possible source of inhibition of membrane associated PKC activity was the 0.1% (v/v) triton X100 detergent used in the extraction of the enzyme from the cell pellet. It had previously been reported that triton did inhibit the enzyme in other systems (e.g. Donnelly et al. (1985)) and so the effects of varying the concentration of the detergent was examined. It was found that triton concentrations above 0.05% (v/v) did indeed inhibit PKC activity from cell homogenates in a dose dependent manner (data not shown) but that, even when detergent levels were lowered to 0.05% (v/v), no membrane associated PKC activity was found. (Experiments performed on membrane fractions isolated in the absence of chelators indicated that 0.05% (v/v) triton was sufficient to liberate the majority of PKC activity from the membrane pellet.)

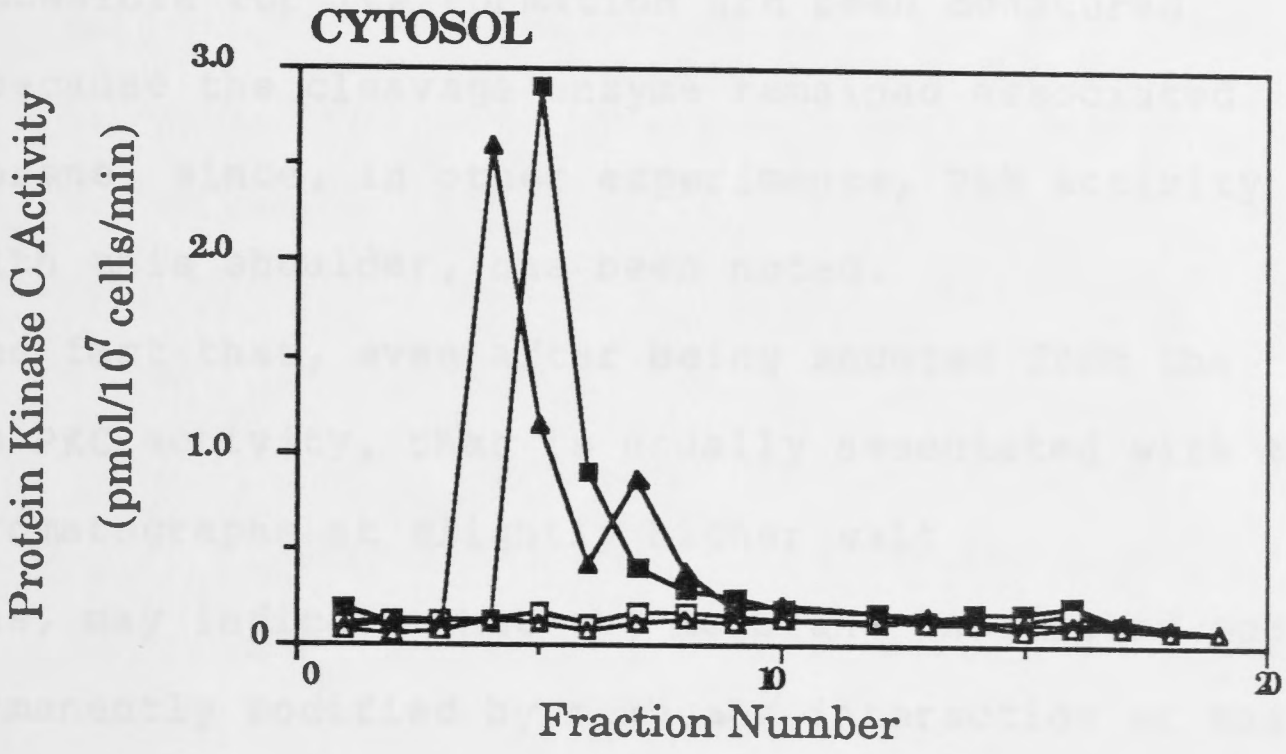
An alternative explanation, which was examined at the same time, was that PKC was being dissociated from the membrane during isolation by the action of divalent metal cation chelators. Such an occurrence was possible, given the reported ability of Ca^{2+} to mediate the translocation of PKC to the membrane in an inside out erythrocyte ghost model and the ability of chelators to reverse this situation, provided

that stabilizers, such as the tumour promoting phorbol esters, are not present (Wolf et al. (1985; 1985a)). It was found that, when cells were isolated in the absence of chelators, a substantial amount of PKC activity was found in the unfractionated membrane extract but that there was no detectable phosphatidylserine-dependent activity in the cytosol. This situation is completely opposite to that seen when either 0.5mM or 5mM EDTA and EGTA are present in the isolation buffer. The possibility, that the absence of PKC activity in the cytosol was due to inhibition of the enzyme by an excess of divalent cations, was dismissed by the re-addition of chelators before assay.

Therefore, on initial inspection, it could be assumed that in vivo, in actively dividing cells, nearly all the PKC activity is associated, albeit loosely, with cellular membranes, with the proviso that the total absence of chelators has not artificially promoted the membrane association of PKC. This conclusion was, however, found to be invalid when activity determinations were made on subcellular fractions after partial purification by DE 52 chromatography (see figure 4.2). It was found that, after fractionation, the cytosolic extracts from cells isolated in the absence of chelators had levels of PKC activity comparable to that in the cytosol of cells isolated in the presence of 5.0mM EDTA and 5.0mM EGTA. The only difference in the profiles was that the EDTA/EGTA cytosol contained an additional shoulder of activity, eluting at a slightly higher salt concentration, which corresponded to the activity peak in the membrane profile from cells isolated without chelators.

FIGURE 4.2

EFFECT OF CHELATORS ON SUBCELLULAR DISTRIBUTION OF PROTEIN KINASE C ACTIVITY



Protein kinase C activity profiles for cytosolic and membrane extracts from the cell hybrid, 39EC13 extracted in buffers with (▲,▲) or without (■,□) EDTA/EGTA and assayed in the presence (■,▲) or absence (□,▲) of phosphatidylserine. Note scale difference between ordinates of cytosolic and membrane profiles. Authenticity of shoulder on the cytosolic peak obtained after extraction with EDTA/EGTA was verified by several experiments.

Interestingly, in this particular profile, the shoulder did not contain the same proportion of PKM as the corresponding membrane associated peak. This is more likely to be because the protease responsible for its formation had been denatured rather than because the cleavage enzyme remained associated with the membrane, since, in other experiments, PKM activity co-eluting with this shoulder, has been noted.

The fact that, even after being shunted from the membrane, the PKC activity, that is usually associated with the membrane, chromatographs at slightly higher salt concentrations, may indicate that the membrane associated pool is either permanently modified by membrane interaction or that the bulk of the cytosolic pool and the membrane associated pool are not interchangeable. As can be seen, there was no PKC or PKM activity found in the membrane extract from cells disrupted with EDTA and EGTA, even after fractionation.

A possible explanation for the apparent lack of PKC activity in the unfractionated cytosol from cells disrupted in the absence of chelators, is that the cells contain an endogenous inhibitor of C-kinase, whose activity is expressed in the cytosol under such experimental conditions. In order to test this possibility, a proportion of unfractionated cytosol was added back to the most active supernatant fraction, and PKC activity re-assessed. When compared to a control, to which the same quantity of extraction buffer had been added, the unfractionated cytosol (10μL/70μL reaction mixture) depressed PKC activity by approximately 50%.

If the observed operation of this inhibitor depends solely on its location in relation to PKC rather than on the

regulation of its activity, then it would have to be predicted that, in the presence of chelators, the inhibitor moves to the membrane, since the cytosolic activity of cells treated in this manner shows no apparent inhibition. There is no positive evidence for this membrane-associated inhibition since under such experimental conditions, PKC leaves the membrane and therefore, moves away from its inhibitor. (This proposal could be tested, however, by examining the effects of adding back a proportion of the membrane extract, from cells extracted with chelators, to active cytosolic fractions). An alternative, perhaps more likely, explanation is that the activity of the inhibitor(s) is dependent on calcium or other divalent cations, explaining why cytosolic fractions from cells isolated in the presence of chelators are free from such inhibition. This is in agreement with work by McDonald and Walsh (1986), in rat brain cytosol, which indicated the presence of 12kDa and 17kda Ca^{2+}-binding proteins that inhibited PKC activity and which were proposed as potential regulators of C-kinase activity, at least in brain, at elevated $[Ca^{2+}]_i$ levels. A complete characterization of the nature of the inhibitory activity in the hybrids, to determine whether it represents a counteractive phosphatase or a specific inhibitor of PKC, will be a worthwhile future investigation.

4.C.3 PHORBOL DIESTER BINDING TO INTACT CELLS:

4.C.3.1 Characteristics of Phorbol Diester Binding

In order to determine whether the perceived decrease in PKC activity in the tumourigenic cell lines was due to a

reduction in the amount of enzyme present, the levels of cellular PKC were quantitated by monitoring tritiated phorbol diester binding in intact cells.

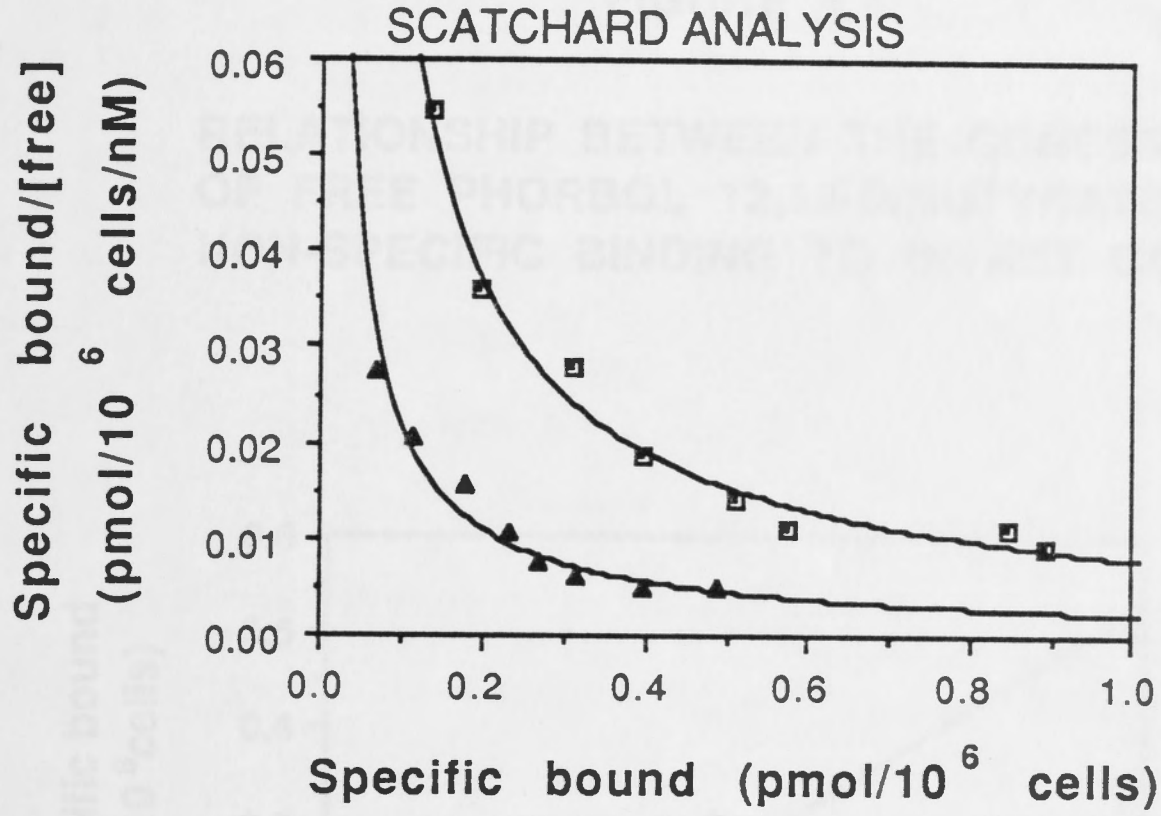
[^{3}H]-phorbol 12,13-dibutyrate (PDBu) was used because, although it is less potent than PMA as a tumour promoter, it is chemically more polar and so can be more readily removed from the cell, resulting in lower levels of non-specific binding (Blumberg et al. (1984)). It has been well documented that the biologically active phorbol esters, including PDBu, promote the translocation of PKC to the membrane (Kraft et al. (1983); Skoglund et al. (1985); Gainer and Murray (1985)) and, thereby, may influence its down-regulation by proteolytic cleavage (Melloni et al. (1985); Tapley and Murray (1985)). However, it was found that when 39EC13 hybrid cells were exposed to 30nM [^{3}H]PDBu at 37°C, the level of phorbol ester which was specifically bound, changed very little between 7 and 68 minutes, indicating that the interaction of phorbol ester with its cellular receptor(s) is rapid and that the apparent rate of down-regulation of PKC in these cells is slow. It could be argued, that the reason for the absence of an observed drop in [^{3}H]-PDBu binding over the one hour period, is because the majority of specific [^{3}H]-PDBu binding sites do not correspond to PKC. However this is unlikely as all evidence points to PKC being the major PDBu binding site (Nishizuka (1984)).

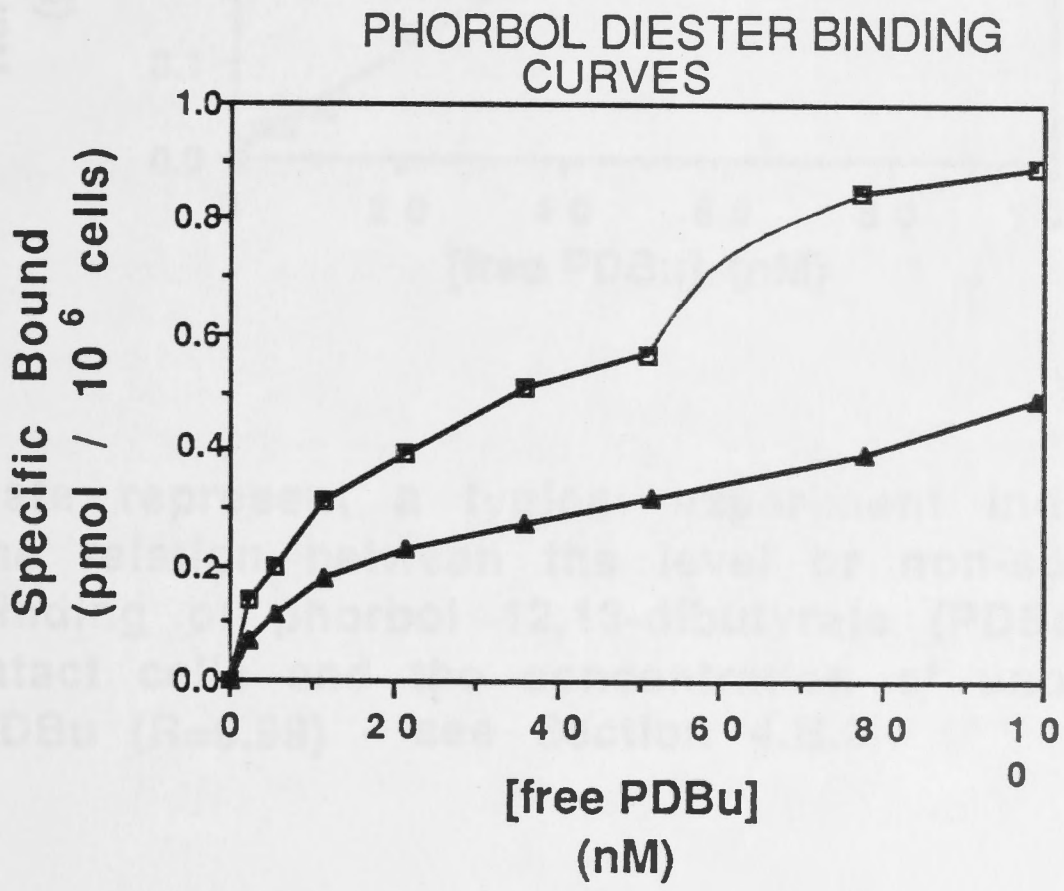
In addition, a number of experiments monitoring the *in vitro* [^{3}H]-PDBu binding to subcellular extracts and DE 52 column fractions were performed with the hybrids, using a

modified version of the binding assay outlined in (Homma et al. (1986)). Quantative results for these investigations are not presented and were difficult to interpret because of extremely low levels of apparent specific binding in these dilute samples against a variable background, which was tentatively attributed to the glass fibre filters used in the assay. (It should be noted that the binding studies are made with ^{3}H label which has a much lower specific activity than that of ^{32}P label used in the activity assays. It should also be noted that binding is a one-to-one event with each molecule of PKC, whereas each enzyme molecule may phosphorylate several peptide substrates in the course of an assay.) Nevertheless, when column fractions from cytosolic extracts were analysed in order to assess their ability to bind PDBu, it was found that a significant and reproducible peak of specific [^{3}H]-PDBu binding co-chromatographed with PKC activity. No additional peak was present. Although this does not negate the possibility that a PDBu-binding protein eluted prior to application of the salt gradient, it does indicate that PDBu binds to PKC and that PKC is the major negatively charged component capable of binding PDBu in the cytosolic extracts.

The number and affinity of PDBu binding sites in the different cell types were examined by determining the levels of specific binding at different concentrations of [^{3}H]-PDBu. Binding curves and subsequent Scatchard analysis for the hybrid pair, $Cn_2B_1Col_1$/5A7mp, are presented in figure 4.3. As can be seen from the Scatchard plots (specific bound/[free] vs specific bound), the points do not fall on a single line but

FIGURE 4.3 BINDING OF PHORBOL 12,13-DIBUTYRATE TO INTACT CELLS: SCATCHARD ANALYSIS AND BINDING CURVES

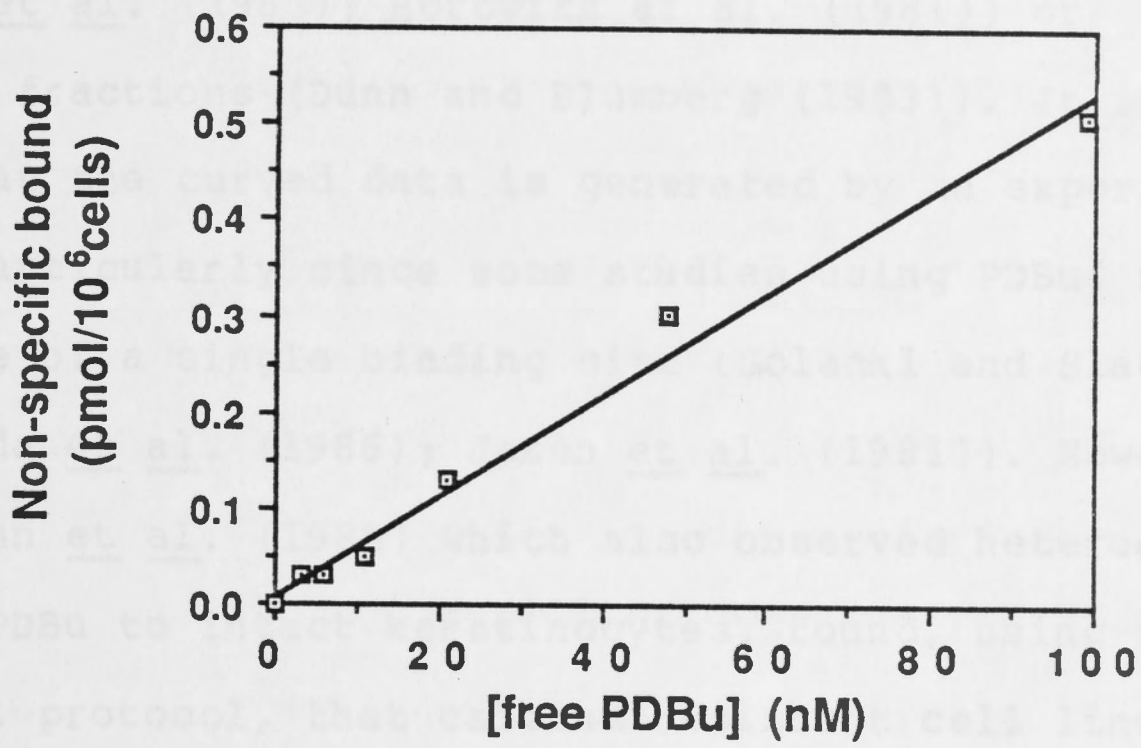




Figures representing the characteristics of specific binding of phorbol 12,13-dibutyrate (PDBu) to the intact transformed (■) and tumourigenic (▲) hybrid pair, Cn $_2$B$_1$ Col$_1$ /5A7mp. Specific binding was calculated as outlined in 3.B.3.

FIGURE 4.4

RELATIONSHIP BETWEEN THE CONCENTRATION OF FREE PHORBOL 12,13-DIBUTYRATE AND ITS NON-SPECIFIC BINDING TO INTACT CELLS



Data represent a typical experiment indicating the relation between the level or non-specific binding of phorbol 12,13-dibutyrate (PDBu) to intact cells and the concentration of unbound PDBu (R=0.99) - see Section 4.B.3

are better described by a curve, from which tangents have been drawn. (This was true for all cell pairs examined although it was less obvious in the 39EC13 cell line which could, perhaps, have been described by a linear relation). This type of plot indicates that there is heterogeneity in PDBu binding to the cells, a finding which has also been reported in a number of other cell systems using intact cells (Dunn et al. (1985); Greenebaum et al. (1983); Horowitz et al. (1981)) or subcellular fractions (Dunn and Blumberg (1983)). It is possible that the curved data is generated by an experimental artifact, particularly since some studies using PDBu, report the presence of a single binding site (Solanki and Slaga (1981); Chida et al. (1986); Jaken et al. (1981)). However, a study by Dunn et al. (1985) which also observed heterogeneous binding of PDBu to intact keratinocytes, found, using the same experimental protocol, that calcium resistant cell lines possessed a single binding site, arguing that heterogeneity was biological rather than experimental. If this heterogeneity is real, it could be generated in a number of ways. It may indicate 2 or more unrelated binding sites indicating that PKC is not, in fact, the only specific cellular receptor for PDBu. Alternatively, the data could represent a continuum of different affinity states of the one receptor. A study by Blumberg et al. (1984), has indicated that variation in phospholipid composition or DAG content of the lipid environment surrounding PKC, alters the affinity of the enzyme's binding to PDBu. Therefore, different pools of PKC, varying in size and membrane location, could conceivably

generate a plot similar to that obtained. A third possibility concerns the different isozymes of PKC. Characterization of their PDBu binding have not yet been reported. If they differ then the heterogeneity could be generated by distinct, but related sites.

Although, given the current information, it is not possible to distinguish between these possibilities, results will be discussed in terms of high and low affinity binding sites based on the dissociation constants for PDBu calculated from Scatchard analysis (See Table 4.2).

A dissociation constant (kd) is the inverse of an association constant (ka) and, therefore, has an inverse relationship to binding affinity. It is calculated as the negative inverse of the slope of the tangent from the Scatchard plot. The kd values for the high affinity site range from 2.6 to 15.3nM. This is in agreement with other reports including those in which only a single PDBu binding site was observed (see Nishizuka (1984)). In addition, studies with purified PKC indicated that PDBu bound with a kd of 8nM (Kikkawa et al. (1983)), indicating that the high affinity site is likely to correspond to PKC. Values for the low affinity sites are less accurate as they are generally obtained from fewer points on the curve. The number of sites associated with this type of binding greatly exceed (at least 3-fold) those of the high affinity sites.

TABLE 4.2

PHORBOL DIESTER BINDING TO INTACT CELLS

Cell type	PDBu binding			
	high affinity		low affinity	
	k_d(nM)	n(sites/cell)	k_d(nM)	n(sites/cell)
5E	3.2	$2.0x10^5$	66.7	$9.0x10^5$
5L	2.6	$1.3x10^5$	50.0	$6.9x10^5$
39EC13	15.3	$4.3x10^5$	100.0	$15.3x10^5$
ESH39	12.5	$2.7x10^5$	33.3	$7.2x10^5$
$Cn_2B_1Col_1$	12.5	$3.9x10^5$	200.0	$16.3x10^5$
5A7mp	11.8	$2.2x10^5$	200.0	$7.5x10^5$
MRC-5	6.7	$5.4x10^5$	100.0	$16.3x10^5$
$D98AH_2$	4.2	$1.6x10^5$	66.7	$8.4x10^5$

Scatchard analysis of the binding of PDBu to intact cells was consistent with the presence of more than one affinity binding site (designated high and low). From Scatchard plots (see Figure 4.3) the dissociation constants (k_d) and the number of binding sites per cell (n) were obtained for each class of binding site in the non-tumourigenic and tumourigenic cells. Values presented are for individual experiments (cell pairs were assayed concurrently).

4.C.3.2 The Relative Number of Higher Affinity Binding Sites in the Transformed and Tumourigenic Hybrids and Parental Lines

When the number of high affinity sites for the different cell types were compared, it was found that, in each case, the non-tumourigenic cell lines have more sites per cell than their tumourigenic partners. All values are in general agreement with those reported previously in other cell types (Dunn et al. (1985)). In order to further examine the relative binding of PDBu by the different cell types, multiple determinations were made at a single PDBu concentration (50nM).

Relative values are given in Table 4.3 accompanied by values for relative numbers of binding sites, obtained from Scatchard analysis (over the full range of concentrations) and relative activity values calculated in section 4.B.1. The values obtained with the single point binding agreed well with the Scatchard figures, with the exception of the MRC-5/D98AH$_2$ pair. This was due to marked variations in the MRC-5 values, also noted in the activity data (table 4.1A), which may be related to the age of the fibroblasts. The D98AH$_2$ HeLa derivatives were the most consistent of all lines examined in both activity and phorbol diester binding measurements.

4.C.3.3 Comparison of Relative Protein Kinase C Activity and Phorbol Diester Binding

As seen from Table 4.3, the relative levels of high affinity binding sites are in closer agreement with the total relative PKC activities than with those activities obtained for membrane-associated PKC. The only exception is the

TABLE 4.3

RELATIVE PHORBOL DIESTER BINDING AND PROTIEN KINASE C ACTIVITY LEVELS IN THE NON-TUMOURIGENIC AND TUMOURIGENIC CELLS

	Relative non-tumourigenic/tumourigenic			
Cell pair	PDBu binding	high affinity PDBu binding	protein kinase C activity total	protein kinase C activity membrane
	A	B	C	D
5E/5L	1.38	1.51	1.69	2.3
39EC13/ESH39	1.66	1.60	2.3	5.0
$Cn_2B_1Col_1$/5A7mp	1.51	1.77	0.45	2.2
MRC-5/D98AH_2	9.20	3.38	6.9	5.6

Relative non-tumourigenic to tumourigenic levels of PDBu binding to intact cells at a single PDBu concentration (50 nM) are given in column A. Such ratios represent a composite of four determinations of specific binding for each cell line. When non-tumourigenic and tumourigenic cells were compared, all sets were found to be significantly different ($p<0.01$) as judged by the Students t-test. Also presented are relative numbers of high affinity binding sites obtained from Scatchard analysis (column B) and relative protein kinase C activity values (column C and D).

$Cn_2B_1Col_1$/5A7mp pair which differed from other activity measurements, in that the cytoplasmic activity of the tumourigenic 5A7mp line exceeded that of its transformed partner.

The extent to which phorbol diesters promote the translocation of PKC to the membrane has not been documented in the somatic cell hybrids. However, it has been reported that phorbol esters have an absolute requirement for PS and Ca^{2+} in order to bind PKC (Tanaka et al. (1986)) and, therefore, it is inferred that the enzyme molecules which bind [^{3}H]-PDBu must have some interaction with the membrane. A comparison of the relative activities and relative binding data, suggest that the majority of PKC is translocated to the membrane by PDBu, although the relative number of PDBu-binding sites per cell are, in most cases, lower than the relative total activity values. This may be because, in addition to reduced amounts of PKC, the activity per molecule may also be reduced in the tumourigenic cells.

Alternatively, there may be a limit to the availability of suitable membrane binding sites and therefore the measureable differences in the higher affinity PDBu binding sites are limited by differences in membrane area. This idea is supported by the observation that the relative PDBu binding sites more closely reflect relative membrane palmitate levels, calculated in chapter 3 (table 3.2) than the total activity valves. This is not to suggest however, that relative PDBu-binding should be corrected for relative membrane area since, given the predominantly cytoplasmic location of PKC

activity in the hybrids, such a relationship would be false. (When relative high affinity PDBu binding sites/cell are corrected for the more suitable parameter of total cellular protein, the differences noted on a cellular basis are maintained.) A third, as yet untested possibility is that the different isozymes bind PDBu with different affinities such that the different forms do not contribute proportionally to total high affinity binding. Therefore, if the differences in PKC activity in the tumourigenic and non-tumourigenic cells is generated by a selective decrease in an isozyme which does not interact strongly with PDBu, then activity differences would not be reflected by the PDBu-binding studies.

If it is assumed that the affinity PDBu binding site represents total cellular PKC and that PDBu and PKC bind in a 1:1 stoichiometry (Nishizuka (1984)) then, by relating the activity data (pmol substrate phosphorylated/ min/10^6 cells) to the number of PKC molecules per cell, it is possible to determine a value for PKC activity/ molecule of enzyme. In the case of MRC-5 (the normal fibroblast line), this value is 2.34×10^{-10} pmol/min/ molecule. House et al. (1987) have reported velocity values for purified rat brain PKC of 1-2 μmol/min/mg protein using synthetic substrates, including GS(1-12) which was used in the present study. Assuming a molecular weight for PKC of 76 000 da (Parker et al. (1986)) these velocities correspond to values of $1.26\text{-}2.52 \times 10^{-10}$ pmol/min/molecule, further supporting the contention that the high affinity phorbol diester binding sites in this study correspond to PKC.

4.D RAMIFICATION OF RESULTS:

Both *in vitro* PKC activity studies and phorbol ester binding studies indicate that the tumourigenic cells have reduced levels of PKC per cell and that this difference, in terms of activity, is accentuated at the membrane, presumably the point at which external controlling stimuli impinge on the cell (Kikkawa and Nishizuka (1986)). When assessing the significance of this finding, it is important to consider whether the observed change is merely a non-specific consequence of the assumption of the tumourigenic state and, more importantly, what role it has in maintaining the tumourigenic phenotype.

Within the milieu of intact cells, it is conceivable that any differences in activity, between the transformed and tumourigenic lines, could be attributed to a more generalized change within the cell; such as an alteration in the properties of the membrane. Such an alteration could generate a number of generalised effects, including a change in PKC activity without any alteration in the PKC molecule, *per se*. However, in the present study, PKC activity was assessed using an *in vitro* assay where the enzyme from both cell types was examined under the same conditions. In addition, the amount of PKC per cell, as judged by PDBu binding, was reduced in the tumourigenic lines, suggesting that the perceived decrease in enzyme activity is due, at least in part, to a change in the level of the enzyme itself, further implying that the change in PKC activity is not just a consequence of some other primary alteration.

It is important, therefore, to consider what ramifications a reduction in this enzyme may have on cell growth. PKC activation, in relation to cellular growth, is most often associated with the generation of a mitogenic signal (Berridge (1987a)). However, as more information concerning the function of PKC is being collected, it is becoming clear that the role of this enzyme in cellular proliferation is highly complex, with activators of PKC often mediating apparently opposing actions, depending on the duration of stimulation (Davis et al. (1985)), the cell type examined (Forsbeck et al. (1985)) and the growth state of the cell (McCaffrey and Rosner (1987)). For example, short term exposure of A431 carcinoma cells to PMA or the synthetic DAG, sn-1,2-dioctanoylglycerol (DiC_8) causes inhibition of high affinity binding of epidermal growth factor (EGF) to its receptor with a subsequent decrease in the receptor kinase activity. However, when quiescent fibroblasts are exposed for a number of hours to PMA or DiC_8, these PKC operators operate synergisticly with submitogenic levels of EGF to stimulate DNA synthesis (Davis et al. (1985)).

Perhaps some of the observed paradoxes can be explained by the finding that the long term treatment of cells with tumour promoting phorbol esters, leads to the depletion of total cellular PKC (Fabbro et al. (1986); Gainer and Murray (1985) and see Borner et al. (1988)) rather than its constitutive activation. Nevertheless, there is still no unifying role for PKC in the mediation of cell growth. For example, it has been indicated that, in some cell types, PMA

induced inhibition of cell growth is due to a decrease in cellular PKC (Gescher and Reed (1985)), whereas in others, it appears that growth inhibition is associated with the activation of this enzyme and that down-regulation of PKC plays no part in the growth inhibition (Issandou and Darbon (1988)).

Another example of the conflicting actions of PKC concerns its role in differentiation. Although PKC activation has been shown to inhibit cell differentiation initiated by hormones and other agents (Shinohara et al. (1985)), it has also been shown to induce differentiation in several cell systems (Homma et al. (1986)). This is interesting in the context of this study, particularly if differentiation is considered to be an alternative, if not opposing process, to proliferation (see general introduction: section 1.D.2). A study (Balazovich et al. (1987)), investigating the activity and cellular distribution of PKC during dimethylsulfoxide (DMSO) or hypoxanthine induced differentiation of murine erythroleukemia cells has indicated that, after addition of inducers, PKC undergoes a rapid and prolonged association with the membrane, accompanied by a gradual decline in total PKC activity over the ensuing 5 days. Moreover, exposure of HL-60 cells to the differentiation inducers, DMSO or retinoic acid, caused at least a two-fold increase in all 3 isozymes of PKC (α, β and γ) in the HL-60 cells (Makowske et al. (1988)). This increase in C-kinase activity was seen in both membrane and cytosolic pools with approximately 10% of total PKC activity being membrane associated in both control and differentiating cells.

The findings with the somatic cell hybrids cannot, however, be directly related to the changes seen in either the differentiation studies or those concerning proliferation since both of these latter cases are examining a dynamic situation where PKC activity and distribution are being monitored during the transition from one growth state to another. In the somatic cell hybrids no equivalent transition is occurring during the current study. Therefore, variations in PKC between the hybrids are conceivably associated with the maintenance rather than generation of the cells' unique growth characteristics, in vivo.

It may be that the decreased level of PKC in the membrane of tumourigenic lines represents the decreased ability of these cells to coordinately regulate incoming mitogenic growth signals, delivered by peptides such as EGF (see introduction 1.C.2.4.5). Another possibility is that the reduced pool size in the tumourigenic cells may mean that the membrane pool cannot be sufficiently enlarged in the presence of appropriate inducers to mediate the types of anti-proliferative signals, associated with differentiation, which are necessary to halt continuous cell growth. The answer to these questions awaits not only a clearer understanding of how PKC regulates cell growth, but also, a more specific determination of the nature of the differences in PKC activity in the tumourigenic cells.

The recent discovery of the different isozymic forms of PKC may help clarify the apparently divergent biological actions of PKC and may also help delineate the nature of the

change in PKC activity in the tumourigenic cells. It could be speculated that the decrease in PKC activity in the tumourigenic cells is due to an imbalance or absence of one of the subtypes of PKC, rather than a reduction in the overall activity of the enzyme. It would be of great interest, therefore, to measure the levels of both mRNA and translation products of the three known isozymes of PKC in the normal, transformed and tumourigenic cells. This would serve, not only to uncover any differential isozymic expression in the different cell types, but also to indicate whether the observed decrease in PKC levels are due to decreased synthesis of message, i.e. change in regulation of PKC genes, or increased degradation of the enzyme, i.e. modulation of other proteins which act on PKC itself. The human α, β and γ PKC genes have been mapped onto chromosomes 17, 16 and 19 respectively (Coussens et al. (1986)), none of which correspond to those chromosomes whose loss from the somatic cell hybrids results in the reversion from a transformed to a tumourigenic state. However, it is conceivable that the expression of C-kinase genes is under the control of the tumour suppressor genes (see introduction 1.D.2) whose presence within the cell is necessary to suppress the tumourigenic phenotype. In this context it would be of interest to examine PKC activity in those tumourigenic hybrids that had reverted back to a non-tumourigenic state after the re-introduction of chromosome 11 (Saxon et al. (1986)) to examine whether a decrease in PKC activity had a causal relationship with the tumourgenic phenotype and not merely a consequential association. A more

direct way to test this, particularly if it eventuates that the tumourigenic cells do have decreased levels of one of the isozymes of PKC, would be to introduce PKC genes, coupled to appropriate promotors, into the tumourigenic hybrids and re-test for tumourigenicity *in vivo*. Alternatively it would be of interest to examine the effect, on tumour development in nude mice, of localized administration of activators or inhibitors of PKC activity at, or after, the time of introduction of the non-tumourigenic and tumourigenic hybrids to the animal host.

Preliminary attempts have been made to assess the impact of a change in PKC activity on expression of the tumourigenic phenotype *in vitro*, by examining the level of cytoskeletal organization before and after treatment of cells with activators and inhibitors of PKC. Results indicate that after 30 minutes exposure to PMA, the tumourigenic hybrid, 5L, showed some improvement in its cytoskeletal organization but that the changes were not substantial.

More work needs to be done in characterizing the changes in PKC associated with the expression of the tumourigenic phenotype in the human somatic cell hybrids. A role for PKC in mediating an altered growth state has been inferred by the ability of phorbol esters to activate (Nishizuka (1984)) and possibly down-modulate (Fabbro *et al*. (1987)) this enzyme, and by the ability of oncogenes to raise the steady state levels of DAG (Fleischman *et al*. (1986)). The findings described in this chapter provide further direct experimental support for a role for PKC in tumourigenesis and

additionally have indicated other avenues for future investigations, including the characterization of the PKC inhibitory activity within the cytoplasm of the hybrids, and the subcellular distribution of PKC in intact cells in response to changes in extracellular [Ca^{2+}].

CHAPTER 5:

CONCLUSIONS

The aim of this investigation was to examine the operation of the phosphoinositide signal transduction pathway in the HeLa X fibroblast human somatic cell hybrid system in order to assess whether this pathway has been deregulated in association with the transition from the transformed to the fully tumourigenic growth state.

The binding of a number of growth factors to their receptors has been shown to stimulate phosphoinositide metabolism, with the cleavage of the lipid phosphatidylinositol(4,5)bisphosphate, resulting in the production of the proposed second messengers, inositol(1,4,5)trisphosphate and diacylglycerol (Berridge (1987a)). An alteration in the functioning of this pathway has been reported in association with the assumption of a transformed or tumourigenic phenotype in several cell systems (see section 1.D).

By examining the incorporation of inositol into the total phosphoinositide pool, and by comparing the relative rates of incorporation with the steady state pool sizes in the transformed and tumourigenic cell hybrids, it was inferred that there was no difference in the turnover of phosphoinositides between the different cell types under maximal growth stimulation (10% serum). A comparison of the parental lines gave similar results in terms of the turnover of the

phosphoinositides. However, it was noted that the HeLa and fibroblast cells differed, not only in their growth characteristics, but also in their morphology which was reflected in the size of their total exchangeable phosphoinosidite pools. This prompted the re-examination of the relative phosphoinositide pools after correcting for differences in cell surface area.

When the phosphoinositide levels within the normal, transformed and tumourigenic cells were expressed per unit membrane rather than per cell, it was found that the tumourigenic cells had higher levels of phosphoinositides/unit membrane than did their non-tumourigenic counterparts. Although this deviance was not significant in every case, it was interesting, since, a higher level of phosphoinositides per unit membrane may give the tumourigenic cells a growth advantage. If it could be shown that the additional phosphoinositides were coupled to receptor complexes that are in excess of their phosphosphoinositide effector in the non-tumourigenic cells then the tumourigenic hybrids would be able to generate higher levels of intracellular mitogenic signals for a given concentration of external agonist than their non-tumourigenic partners. However, a comparison of the ability of the different cell hybrids to divide in different serum concentrations, failed to indicate a difference in the growth responsiveness in the cell types. Moreover, when cell hybrid pairs were grown under maximal growth stimulation conditions (10% (v/v) serum) a comparison of the steady state levels of inositol (1,4,5)trisphosphate, indicated that there

was no difference in the levels of this molecule between the hybrids. Therefore it was concluded that, in tissue culture, there was no functional difference in the operation of the phosphoinositide pathway between the transformed and tumourigenic cells.

The absence of a significant difference in the rates of turnover of the phosphoinositides, between any of the cell types, may signify that this pathway is functioning normally in the hybrids. Conversely, it may indicate that it is not functioning at all to mediate serum stimulated proliferation in the actively dividing cells. This latter possibility should not be dismissed, given the recent report that there is no appreciable change in phosphoinositide metabolism during the progression through the cell cycle of Chinese hamster ovary cells, stimulated to divide with serum (Tones et al. (1988)).

However, although the transformed and tumourigenic hybrids have different growth characteristics in vivo, they have very similar growth properties in vitro. (Standbridge et al. (1982)). In addition, it is not until the hybrids have spent several days within the animal host that the full expression of the tumourigenic potential becomes obvious (Stanbridge and Ceredig (1981)). This indicates that the activation or function of the suppressor genes, proposed to be lost in association with the assumption of the tumourigenic phenotype, (Stanbridge (1985)) is not instantaneous in vivo and that its complete operation in vitro is not possible (as assessed by the inability of the non-tumourigenic hybrids to be growth arrested in G_o or to display the differentiated

phenotype of the normal parent). Therefore, although the present study does not support the suggestion that an altered turnover in phosphoinositides is a key difference associated with tumourigenicity, the possibility cannot be eliminated that minor alterations in phosphoinositide metabolism, detected in this study, are amplified by the distinct environment occurring *in vivo*.

One difference between the transformed and tumourigenic hybrids, which was observed in tissue culture, concerned the levels of protein kinase C within the cells. It was found that both the *in vitro* kinase activity, and the high affinity phorbol diester binding in intact cells, was reduced in the tumourigenic hybrids, suggesting that the actual amounts of this enzyme are lower in such cells. This indicates that the change in protein kinase C activity occurred independently of phosphoinositide hydrolysis but that it may affect the outcome of the activation of this pathway. It could be argued that the decreased levels of protein kinase C would lead to a reduction in the availability of positive growth mediators in the tumourigenic lines. However, protein kinase C has equally been shown to mediate many negative effects of growth including, not only inhibition of its own activator-generating system (Kikuchi *et al.* (1987)), but also the inhibition of other growth signals (Bollag *et al.* (1986)) and in some cells, the induction of differentiation (Makowske *et al.* (1988)).

Before making a full assessment of the importance of these findings to the assumption of the tumourigenic state, it will be necessary to assess the magnitude of the difference in

intracellular protein kinase C activity in the intact hybrids, and highly desirable to determine the proportionate expression of the different known isozymes of C-kinase within the transformed and tumourigenic cells. Even with this information, it could be argued that the observed decrease in C-kinase levels is a non-specific consequence of tumourigenesis which plays no part in the progression or the maintenance of the tumourigenic state. However, it could equally well be proposed that PKC plays a central role in the regulation of cell growth, and that a reduction in the levels of this enzyme actively contributes to the tumourigenic phenotype. This could no doubt be tested directly using the tools of molecular biology, as outlined in chapter 4. Intellectually, this idea is supported from the current information concerning the transforming oncogenes. Oncogene products have been shown to mimic the function of growth factors (*sis*), growth factor receptors (*erb*-B), and GTP-binding proteins (*ras*). They can enhance phosphoinositide turnover (*ros*, *src*, *ras*, *fms*, *fes*) and are highly related to the cellular genes which are switched on by second messengers (*myc*, *fos*). In fact, they appear to have mimicked every stage in the generation of positive mitogenic signals. Yet none of the known oncogenes have sequence homology to protein kinase C (Parker *et al*. (1986)). This would make sense if the primary role of this kinase is the regulation rather than the promotion of growth.

It is proposed, therefore, that the observed decrease in protein kinase C levels in the tumourigenic somatic cell hybrids, compared with their transformed partners,

actively contributes to the tumourigenic growth state and that at least one protein kinase C gene is a target for the tumour suppressor gene(s) whose presence within the cell is necessary for controlled growth within the animal. It remains to be seen whether the additional protein kinase C activity in non-tumourigenic cells helps control growth by promoting differentiation or modulating proliferation. Whatever the outcome, the findings will further the understanding of the role of protein kinase C in cell surface signal transduction.

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APPENDIX

COMPOSITION OF MEDIA

(a) Phosphate Buffered Saline (PBS)

137 mM	NaCl
7 mM	$Na_2HPO_4.2H_2O$
3.5 mM	$Na\ H_2PO_4.2H_2O$

(b) Minimal Essential Medium without glucose (MEM-G)

116 mM	NaCl
1.36mM	$CaCl_2$
5.4 mM	KCl
1.0 mM	$MgSO_4.7H_2O$
26.2mM	$NaHCO_3$
1.0 mM	$NaH_2PO_4.2H_2O$

ABBREVIATIONS

AA	arachidonic acid
AEV	avian erythroblastosis
AMP,ADP,ATP	adenosine mono-, di-, and triphosphate
c-AMP	adenosine 3',5'-cyclic monophosphate
cDNA	complementary DNA
c-GMP	guanosine 3',5'-cyclic monophosphate
cIns(1:2)P	inositol cyclic monophosphate
CMP,CDP,CTP	cytidine mono-, di-, and triphosphate
DAG	diacylglycerol (*sn*-1,2 configuration unless otherwise stipulated)
DEAE-cellulose	diethylaminoethyl cellulose
DiC_8	*sn*-1,2-dioctanoylglycerol (synthetic DAG)
DMS0	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EGF	epidermal growth factor
ER	endoplasmic reticulum
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol bis(β–aminoethylether)-*N,N,N',N'*-tetraacetic acid
FMLP	formyl-methionyl-leucyl-phenylalanine
FSV	feline sarcoma virus
G_0, G_1, G_2	stages or temporal gaps in the cell cycle
gly	glycine
GMP,GDP,GTP	guanosine mono-,di- and triphosphate
G_p, G_s, G_i	GTP-binding proteins (also N_p, N_s, N_i)
GTP γ-S	non-hydrolysible analogue of GTP
HPLC	high performance liquid chromatatography
IFβ	interferon type beta
ILF-1	insulin-like growth factor
InsP, $InsP_2$,$InsP_3$	inositol mono-, bis-, tris-, tetrakis-,
$InsP_4$, $InsP_5$, $InsP_6$	pentakis-, and hexakisphosphate, respectively. Numbers in parenthesis denote regiochemistry
$[ion]_i$	free intracellular molar concentration of ion denoted
$[ion]_o$	free extracellular molar concentration of ion denoted
mRNA	messenger ribonucleic acid
OAG	1-oleoyl-2-acetylglycerol (synthetic DAG)
PDBu	phorbol 12,13-dibutyrate
PDGF	platelet-derived growth factor
pH_i	intracellular pH

PI, PIP, PIP_2	phosphatidylinositol, phosphatidylinositol mono-, and bisphosphate. Numbers in parenthesis denote regiochemistry.
PKA	protein kinase A *viz* c-AMP-dependent protein kinase
PKC	protein kinase C
PKM	protein kinase M
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonyl fluoride
PPO	2,5-diphenyloxazole
PS	phosphatidylserine
TCA	trichloroacetic acid
TGFβ	transforming growth factor beta
TNF	tumour necrosis factor
v-*fes*, v-*fms*, v-*ros*, v-*ras*, v-*src*,v-*myc*, *v-fos*,*v-erb B*	oncogenes
c-fes, v-fms etc	proto-oncogene